DETECTION AND PHENOTYPIC CHARACTERIZATION OF DRUG (METABOLITE) - SPECIFIC T-CELLS FROM PATIENTS WITH DRUG-INDUCED SKIN AND LIVER INJURY AND HEALTHY DONORS

This thesis is submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy by

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Declaration

I declare that the work presented in this thesis is all my own work and the data presented in this thesis was obtained from human sample *in vitro* experiments carried out by myself in Molecular & Clinical Pharmacology centre/Institute of Translational Medicine/University of Liverpool. And has not been submitted for any other degree.

…….

Khetam Alhilali

B.VM&S, M.Sc. (Pharmacology)

To my parents

To my sister (sohar)

To my family

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List of publications and presentations.

Below are the published papers and manuscripts prepared for peer-review in scientific journals and published conferences presentations that have originated from this thesis.

Publications:

1. Khetam Ali Alhilali, Zaid Al-Attar, Andrew Gibson, Mario Monshouwer, Jan Snoeys, B. Kevin Park, Dean J Naisbitt. Characterisation of healthy donor-derived T-cell responses specific to telaprevir diastereomers. 2018. Toxicol Sci.

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National and international conferences.

1. Khetam Alhilali, Zaid Al-Attar, Andrew Gibson, B. Kevin Park, Dean J Naisbitt. Characterisation of Healthy Donor-derived T-cell Responses Specific to Telaprevir Diastereomers. The EAACI 8th Drug Hypersensitivity Meeting (DHM), 19 - 21 April 2018, Amsterdam, the Netherlands. (Poster presentation).
2. Khetam Alhilali, Qing Zhao, Abdulaziz Alzahrani, Mubarak Almutairi, Monday Ogese, Andrew Gibson, David A. Ostrov, Furen Zhang, Dean J. Naisbitt. Characterization Of Dapsone- And Nitroso-Dapsone-Specific T Cells From Hypersensitive Patients Expressing HLA-B*13:01.The EAACI 8th Drug

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3. Khetam Alhilali, Qing Zhao, Abdulaziz Alzahrani, Mubarak Almutairi, Monday Ogese, Andrew Gibson, David A. Ostrov, Furen Zhang, Dean J. Naisbitt. Characterization Of Dapsone- And Nitroso-Dapsone-Specific T-Cells From Hypersensitive Patients Expressing HLA-B*13:01. Liverpool's National Research Conference. 14th April 2018 (oral presentation).
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List of Abbreviation

AB	Antibody
ACD	Allergic contact dermatitis
ACE	Angiotensin-converting enzyme
ADPKD	Autosomal dominant polycystic kidney disease
ADR	Adverse drug reaction
ADRs	Adverse reactions
ADEs	Adverse drug events
ALP	Alkaline phosphatase
APC	Antigen presenting cell
APC*	Allophycocyanin
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium
BCR	B-cell receptor
BCl6	B-cell lymphoma 6
β_2m	Beta-2 microglobulin
BSA	Bovine serum albumin
CBZ	Carbamazepine
CD	Cluster of differentiation
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLIP	Class II-associated Ii peptide
Cmax	Maximum (or peak) serum concentration that a drug achieves in a specified compartment or test area of the body after the drug has been administered and prior to the administration of a second dose.
CPM	Counts per minute
CSA	Cyclosporine-A
CTLA4	Cytotoxic T-lymphocyte associated protein-4
CTLs	Cytotoxic T-lymphocytes
CXCR	CXC chemokine receptors.
μCi	Curie (symbol Ci) unit of radioactivity

Da	Units of atomic mass (Dalton)
DC	Dendritic cell
DDS	Dapsone
DDS-NO	Dapsone Nitroso
DIHA	Drug induced immune hemolytic anemia
DMSO	Dimethyl sulfoxide
DRESS	Drug reaction with eosinophilia and systemic symptoms
EAACI	European Academy of Allergology and Clinical Immunology
EBV	Epstein-Barr virus
EBVs	B-lymphoblastoid cell lines transformed by Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELIspot	Enzyme Linked Immunospot Assay
ER	Endoplasmic reticulum
FACs	Fluorescence-Activated Cell Sorting
FACS	Flow cytometry
FasL	Fas Ligand
FBS	Foetal Bovine Serum
FITC	Fluorescein isothiocyanate
FoxP3	Forkhead box P3
FSC	Forward scattering
GB	Granzyme B
GM-CSF	Granulocytes-macrophage colony stimulating factor
Gy	Gray (unit) of absorbed radiation
HBSS	Hanks balanced salt solution
HCV	Hepatitis C virus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human Leukocyte Antigen
ICU	Intensive Care Unit

IDILI	Idiosyncratic drug induced liver injury
IFN-γ	Interferon-gamma
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
LPS	Lipopolysaccharide
LTT	Lymphocyte transformation test
M11	VRT-841125/ (TEL. M2).
M-CSF	Macrophage colony-stimulating factor
MHC	Major Histocompatibility complex
MIDRs	Metabolic idiosyncratic reactions
NK	Natural Killer
NSAIDs	Nonsteroidal Anti-Inflammatory Drugs
PAMPs	Pathogen-Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PHA	Phytohaemagglutinin
P-I	Pharmacological interaction of drugs with immune receptors
PRRs	Pattern Recognition Receptors
RPMI	Roswell Park Memorial Institute
SD	Standard Deviation
SFU	Spot Forming Unit
SI	Stimulation Index
SJS	Stevens–Johnson syndrome
SMX	Sulfamethoxazole
SMX-NO	Sulfamethoxazole-nitroso
SSC	Side Scattering
TAP	Transporter associated with antigen presentation

T-bet	T-box transcription factor
TCCs	T-cell clones
TCR	T-cell receptor
TVR (TEL)	Telaprevir (S-diastereomer), (TVR, VX-950).
TEL.M	R-diastereomer
TEN	Toxic Epidermal Necrolysis
TFH	T follicular helper cells
TGF	Transforming Growth Factor
Th	T-helper cells
TLR	Toll-like Receptors
TNF-α	Tumor necrosis Factor Alpha
TNTC	Too Numerous To Count
Treg	Regulatory T-cells
TT	Tetanus toxoid
TVP	Tolvaptan
W/V	Weight/volume
WAO	World Allergy Organization
Zap70	Zeta chain-associated protein kinase

ABSTACT.

Drug hypersensitivity reactions are unpredictable adverse drug reactions. They represent a considerable clinical problem and an obstruction to drug development. Delayed-type drug hypersensitivity reactions targeting skin and/or liver are thought to have an immune pathogenesis with drug-specific T-cells believed to play an important role in the initiation and regulation of tissue injury. The objective of this thesis was to explore the cellular basis of three forms of drug hypersensitivity reaction, namely, dapsone hypersensitivity syndrome, tolvaptan-induced liver injury and telaprevir-induced severe skin injury. The availability of patient and healthy donor PBMC allowed us to (1) clone drug-specific T-cells, (2) explore the phenotype of T-cells involved in different forms of hypersensitivity and (3) characterize mechanisms of drug antigen presentation.

Drug hypersensitivity associated with expression of specific HLA alleles has focussed on the interaction between parent drug and the HLA with no attention given to reactive metabolites. For this reason, we studied HLA-B*13:01-linked dapsone hypersensitivity. PBMC from 6 patients and cloned T-cells proliferated and secreted Th1/2/22 cytokines when stimulated with dapsone (clones: n=395; 80% $CD4^+$ $CXCR3^{hi}CCR4^{hi}$, 20% $CD8^+CXCR3^{hi}CCR4^{hi}CCR6^{hi}CCR9^{hi}CCR10^{hi}$) and nitroso dapsone (clones: n=399; 78% $CD4^+$, 22% $CD8^+$ with same chemokine receptor profile). $CD4^+$ and $CD8^+$ clones were HLA-class II and class I restricted, respectively, and displayed three patterns of reactivity: compound-specific, weakly crossreactive and strongly cross reactive. Nitroso dapsone formed dimers in culture and was reduced to dapsone, providing a rationale for the crossreactivity. T-cell responses to nitroso dapsone were dependent on the formation of a cysteine-modified protein adduct, while dapsone interacted in a labile manner with antigen presenting cells. $CD8^+$ clones displayed an HLA-B*13:01-restricted pattern of activation.

Telaprevir, a protease inhibitor, was used alongside PEGylated interferon-α and ribavirin to treat hepatitis C viral infections. The triple regimen proved successful; however, the appearance of severe skin reactions alongside competition from newer drugs restricted its use. Thus, we used PBMC from healthy donors to investigated whether telaprevir and/or its diastereomer, which is generated in humans, activates T-cells. Drug-specific $CD4^+$ and $CD8^+$ T-cell clones responsive to telaprevir and the R-diastereomer were generated and characterised in terms of phenotype and function. The clones proliferated in a dose-dependent manner and secreted IFN-γ, IL-13, and granzyme B in response to culture with telaprevir and the diastereomer at the same concentrations. The T-cell response was MHC I-restricted and dependent on the presence of soluble drug. Flow cytometric analysis showed that clones expressed chemokine receptors CCR4 (skin homing) and CXCR3 (migration to peripheral tissue) and one of three distinct TCR Vβs; TCR Vβ 2, 5.1, or 22.

Use of the selective arginine vasopressin V2 receptor blocker tolvaptan is associated with the development of liver injury. We recruited PBMC from patients with liver injury to explore whether tolvaptan and/or its two metabolites DM-4103 and DM-4107 activate T-cells. Drug-specific T-cells were detected infrequently and only in a small number of patients. The majority of clones were stimulated to proliferate and secrete cytokines with the metabolite DM-4107 and not the parent drug.

Collectively, these studies have identified drug and drug metabolite-responsive $CD4^+$ and $CD8^+$ T-cells in healthy donors and patients with skin and liver injury. Through the generation of T-cell clones we were able to characterize mechanisms of T-cell activation with parent compounds and synthetic stable and reactive metabolites. Furthermore, we have shown that drugs and reactive metabolites participate in HLA allele linked forms of hypersensitivity.

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Chapter1: introduction and literature review

1 General introduction:

In the modern era, the encumbrance from drug problems became equal to disease problems, especially with an increase in unexpected cases of severe adverse reactions to drugs. Researchers continue to study the mechanisms of drug hypersensitivity, and the reasons why certain individuals are susceptible. To do this, researchers explore the interaction between the human body (especially the immune system) and drugs. In the long term, these specific studies will help to control and prevent more cases of drug hypersensitivity diagnosis. The aim of this introductory section is to explain the basic topics of immunology and pharmacology, and some cases the interaction of drugs in the human body. This provides the framework for my investigation into the cellular basis of drug hypersensitivity.

1.1 Immunology.

Immunology is the study of the body's defence against infection. This science started when Edward Jenner in the 18th century observed that the mild disease of cowpox or vaccinia could confer a protection from the fatal version of the disease. Although Jenner introduced vaccination, he knew nothing about the causative agent. That became known only in the late 19th century, when Robert Koch demonstrated that the disease was caused by specific microorganisms (Murphy, 2012).

1.2. Immune system.

Is a complex network consisting of organs, cells and molecules spread through the body to deal with pathogens, viruses, bacteria and parasites (Chaplin, 2010). The main function of the immune system is a defence against pathogens and prevention of these pathogens from entering the body (Chaplin, 2006). Furthermore, if these pathogens succeed in penetrating the external defence of the body, the immune system will determine which type of response

will occur. The immune response is classified into two areas based on the nature of the pathogen.

- An intracellular pathogen (invades the body cells to reproduce).
- An extracellular pathogen (does not enter the inside of host cells)

1.2.1. Innate and Adaptive Immunity.

The immune system deals with pathogens in different ways, and this response is determined by two components of the immune system:

- **Innate immune response** (natural or native immunity or non-adaptive immune system). In order for the immune system to respond to an invading pathogen, it needs to first identify the threat as "non-self" pathogen. The immune system can distinguish and respond to foreign patterns. (Gallucci and Matzinger, 2001, Maverakis et al., 2015). The innate immune system depends on a limited number of germ line-encoded pattern recognition receptors (PRRs), which detect various structures on pathogens, these structures are termed pathogen-associated molecular patterns (PAMPs). Toll-like receptors are the more important receptors among all the PRRs (Suresh and Mosser, 2013, Mogensen, 2009). The response is rapid as this system does not have to learn /adapt to the variability of the environment. Thus, the innate system responds in the same way when exposed to the same pathogen twice.
- **Adaptive immune response** (antigen-specific adaptive immune response). It is characterised by a slow response, because initial antigen exposure leads to the activation of naïve lymphocytes (Danilova, 2006, Koenderman et al., 2014). After the initial immune response recedes, a small population of long-lived memory T-cells ensure that the host is never infected again by the same pathogen.

Both the innate and adaptive immune responses are involved in the secretion of soluble proteins, receptor-mediated signalling, and intricate cell-to-cell communication. The two components act together to eliminate the pathogen.

1.2.2 The main components of the innate system are:

- **Physical and chemical barriers**:

These include the skin and its layers including mucous membranes. In addition to being a physical barrier, the skin provides low pH. The epithelial tissue can secrete substances to kill the pathogen or weaken its activity (Clark et al., 2007). Chemical barriers include endogenous chemicals substances such as the hydrolytic enzymes within saliva or the low pH of the stomach and the vagina (Parham and Janeway, 2009, Davies, 1997, Kenneth Murphy, 2017).

- **Cellular barriers**.

Natural killer cells, macrophages, monocytes, granulocytes (which include neutrophils, eosinophils and basophils) and dendritic cells are responsible for combatting the invading microorganisms, they can engulf and digest invading pathogens. These cells gulp the pathogens by the mechanisms of pinocytosis, receptor mediated endocytosis, and phagocytosis. Endocytosis is not cell specific and is perhaps carried out by all cells. However, phagocytosis is more cell-specific and results in the ingestion of the particulate as well as the whole microorganism (Murphy and Weaver, 2017).

- **Blood proteins.**

The blood contains members of the complement system and other mediators of inflammation (Abbas et al., 2018, David et al., 2013). The complement system plays an enormous role in fighting infections through several mechanisms which include:

opsonisation, chemotaxis, cell lysis and clumping of antigen-bearing agents (Parham and Janeway, 2009).

- **Innate mechanisms.**

Innate immunity works jointly in inhibiting the invasion of a pathogen or eliminating it, preventing infection. Furthermore, innate immunity fights the pathogen until the slower adaptive immune response can be activated (Mak et al., 2014). The innate immune system recognises pathogens by patterns. These include lipopolysaccharides (LPS), mannose, fructose, teichoic acid and N-formyl peptides. These common microbial patterns are called Pathogen-Associated Molecular Patterns (PAMPs) (Khan, 2016). In contrast, there are special molecules known as Toll like receptors (TLRs) that are expressed by the cells of the innate immune system. These receptors are responsible for recognising PAMPs (Alegre et al., 2008). Gamma Interferon (IFN-γ) and Tumour Necrosis Factor-alpha (TNF-α) are important molecules which are secreted from innate cells such as Natural Killer (NK) Cells. They provide a connection between the innate and adaptive responses (Eagar and Miller, 2008, Abul K Abbas, 2015). Moreover, the recognition of the pathogen by the Toll-like receptors (TLRs) expressed on cells residing close to the infection site leads to secretion of chemokines, which are soluble proteins that have a special chemotactic role for immune cells (Bachmann et al., 2006).

1.2.3 Adaptive immune system.

The adaptive immune system seeks to protect humans from death by pathogens. The major difference between the innate immune system and the adaptive immune system is that adaptive responses are highly specific and provide long-lasting protection (Alberts et al., 2002). The adaptive immune response is so called because it adapts to the first exposure to foreign antigen. This is often referred to as the primary response to antigens. When the body is exposed to the same pathogen, the exposure will reproduce another type of response

known as the secondary response. Adaptive immune responses are achieved by the white blood cells, which are also called B and T lymphocytes. These mediate cells humoral and cellular components of the adaptive immune response, respectively. These mechanisms are highly specific and are only activated in the presence of a single antigen (Khan, 2016, Alberts, 2002, Kutteh et al., 2014).

- **Humeral mechanisms (antibodies).**

Antibodies (also known as immunoglobulins) are glycoproteins produced by activated B-cells during the immune response. Before maturation, B-cells express special receptors on their surface, which are specific for antigens (immunoglobulin molecules). After the B-cell becomes activated, it matures, and then it differentiates into a plasma cell which secretes a large number of soluble immunoglobulins into the extracellular fluid. This type of secretory molecule in a soluble form is known as an antibody. This antibody will be specific for the same antigen that initially activated the B-cell. However, these antibodies will support phagocytes and remove the pathogen in a process known as opsonisation. The antibody molecule is a Y-shaped structure, consisting of two heavy (H) and two light (L) chains. Depending on the function of these structures, it is divided into variable (V) domains which are specific to antigens and constant (C) domains which designate effector functions (Male et al., 2012). Moreover, there are five general classes of heavy chain C domains, each class gives a definition of the immunoglobulin isotypes such as; IgM, IgG, IgA, IgD, and IgE. Each one of these isotypes is divided into subclasses. For example, IgG divides into four subclasses, IgG1, IgG2, IgG3, and IgG4 (Leder, 1982, Tonegawa, 1983, Schroeder and Cavacini, 2010). Each antibody has an individual specificity.

- **Cellular mechanisms.**

The main cells involved in cellular immune responses are B and T lymphocytes. The classification and function of B and T lymphocytes are discussed below.

1.2.4 The immune system cells.

All the cellular elements of blood, including the red blood cells, platelets and the white blood cells, derive from the same progenitor or precursor cells which are known as hematopoietic stem cells, that are found in the bone marrow (Janeway, 2001, Lai and Kondo, 2008). These various cell types have specific roles (Khan, 2008), which are explained in the following paragraphs.

1.2.4.1 The lymphoid lineage.

Lymphocytes are highly discriminatory white blood cells and they are the main defenders in adaptive immune response. They achieve their role through two broad types of immune responses, either antibody responses or cell-mediated immune responses (Kutteh et al., 2014, Alberts et al., 2002).

1.2.4.1.1 B-cell:

B lymphocytes constitutes 20-25% of total lymphocytes in the blood (Tomasek et al., 2011). B-cells distinguishe the foreign antigen directly by a molecule called the B-cell receptor (Zou et al., 2017). B-cell defence against pathogens occurs by various mechanisms, all of these mechanisms depend on antibodies. During the development period, the immature B-cells express Cluster of Differentiation (CD) 20. These cells have the unique function to generate antibodies, which take place after binding to antigens. The B-cell then converts into a plasma cell or memory cell depending on the signals it recieves (Hoffman et al., 2016).

- There are two pathways for B-cell activation (Baumgarth, 2003, Bretscher and Cohn, 1970);

- **T-independent B-cell activation.**

When the antigens such as bacterial lipopolysaccharides, interact with B-cells, they require a second signal which triggers TLRs for full activation. These signals stimulate B-cells to

proliferate and produce IgM antibodies. Generally in this activation pathway, the IgM antibodies have a low affinity, hence it is an innate immune stereotyped weak response. As it is demonstrated in the next section, T-cells play a crucial role in the B-cell activation process (Figure 1.1 A) (Baumgarth, 2003, Vos et al., 2000).

- **T-dependent B-cell activation.**

In this pathway, antigens will bind to B-cells with B-cell receptors (BCRs), providing the 1st signal to the inactive B-cells. Full activation depends on various other immune cells such as antigen presenting cells (APCs), dendritic cells (DCs) or macrophages which present the antigen to a special type of T-cell known as Th2 secreting T-cells. These cells secrete cytokines such as Interleukin (IL) 4, 5 and 13, which provide the 2nd signal for B-cell activation (Fazilleau et al., 2007). After activation the B-cell, will be converted into plasma or memory cells. The plasma cells have the potential to produce IgG, IgA and IgE antibodies. This process is referred to as class switching because it will change the characteristics of the humoral response. These antibodies are highly specific and exert different functions (Allen et al., 2007). The role of T-cells in B-cells activation is summarized in Figure 1.1 B (Male et al., 2012).

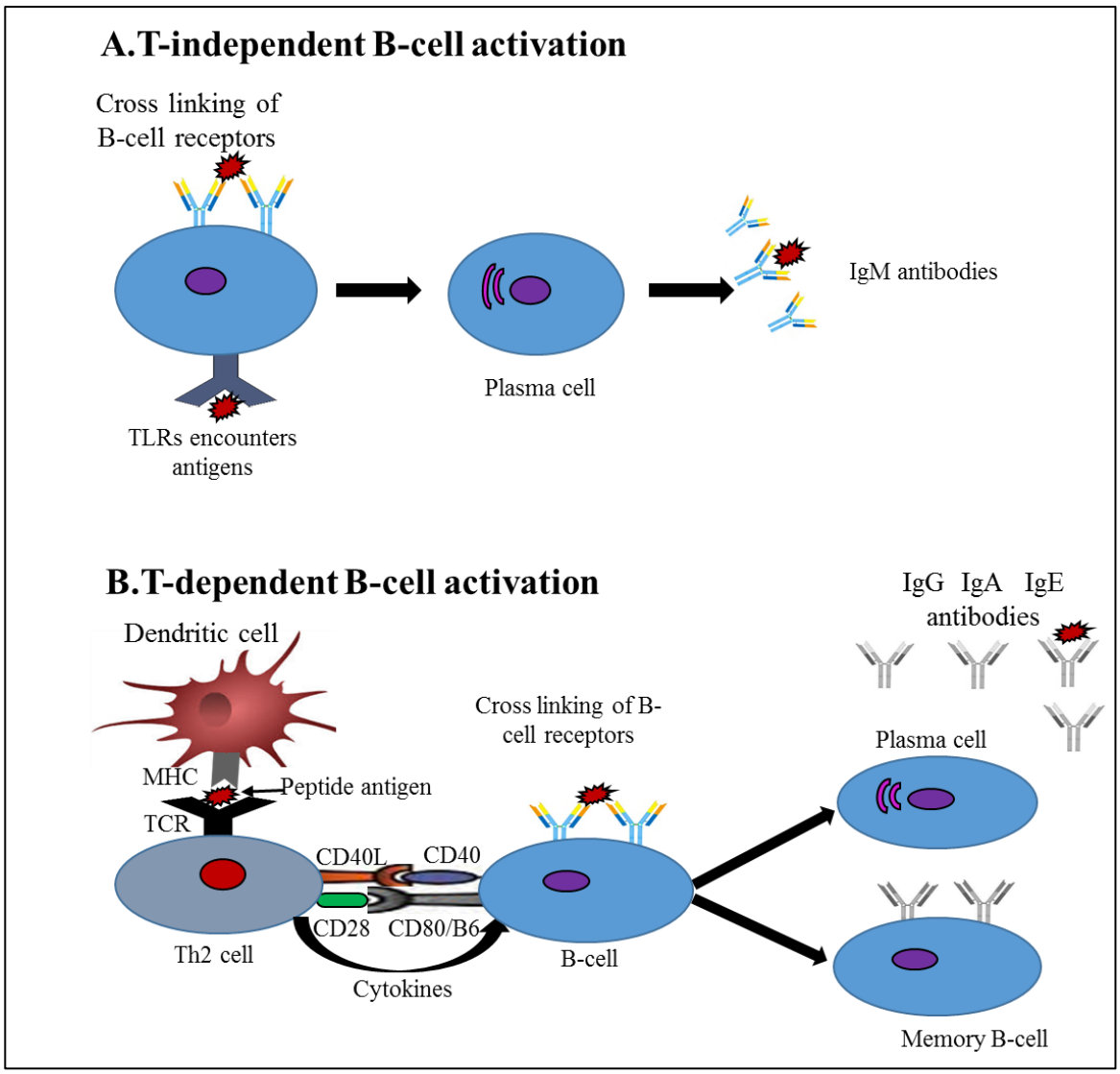

Figure 1-1 (A) T-independent B-cell activation: B-cell stimulation by antigens (bacterial antigens like the polysaccharides) directly through antigen mediated cross-linking of BCRs (1st signal). Also, antigens bind with TLRs (2nd signal). This activation leads to maturation to the plasma cell, and then these cells will continue to produce IgM antibodies.

(B) T-dependent B-cell activation: B-cells receive a 1st signal from antigen mediated cross-linking of BCRs. Furthermore, T-cells are activated by the antigen presenting cells that express CD28, CD40L and secrete Th2 cytokines (2nd signal). This will lead to B-cell maturation and the generation of plasma cell or memory cells.

1.2.4.1.2 Natural Killer cells (NK cells).

NK cells are lymphocytes of the innate immune system. NK cells represent 15% of blood lymphocytes. NK cells develop in the bone marrow, similar to B-cells. However, NK cells can also develop in lymph nodes and the liver (David et al., 2012, Mandal and Viswanathan, 2015). NK cells depend on the cytokine IL-15 for their normal development and maintenance

(Khan, 2008). Their main biological function is recognising and killing virally-infected and neoplastic cells but these cells are also able to respond to protozoa and bacteria (Yokoyama et al., 2004, Mandal and Viswanathan, 2015). The NK cells are part of the innate response, and respond rapidly. The response is not previous sensitisation. (Vivier et al., 2008, Vivier et al., 2011). NK cells mainly express $CD56^{+}$ (CD3- cells); however, the expression of this molecule differs on the individual cells. Among the NK cells in blood, 90% of the NK cells are CD56 low (CD56 low). These cells contain granules and are efficient killers. The other 10% of blood NK cells are CD 56 high. The CD56 high NK cells do not have granules to complete their function as killer cells. But they respond to target cells by producing important substances in immune response like Th1 cytokines (Rebuli et al., 2018, David et al., 2012, Burleson et al., 2015, Van Acker et al., 2017). These cells are not restricted to MHC-dependent antigen presentation. They are activated by glycolipid antigens that interact with CD1d (Godfrey et al., 2005). **The CD1d is a major-histocompatibility-complex (MHC) class-I-like molecule, which is responsible for presenting self-lipid and non-self-lipid antigens to NK cells** (Oleinika et al., 2018, Godfrey et al., 2005). To complete the protection role NK cells have the ability to detect the foreign cells in non-MHC self-molecules. NK cells use inhibitory receptors to measure the loss of constitutively expressed self-molecules on the surface of target cells. When NK cells are facing target cells, the NK cells recognise missing self-MHC molecule on the target cells (Karre et al., 1986, Bix et al., 1991). That means in case of down regulated or absent self-MHC, the NK cells become active and kills the target cells due to loss of the inhibitory signals (Medzhitov and Janeway, 2002, Storkus et al., 1987, Vivier et al., 2008). The NK cells have important TLRs (Lemaitre et al., 1996), which upon activation will encourage NK cells to secrete IFN-γ and will enhance cytotoxicity (Vivier et al., 2008, Souza-Fonseca-Guimaraes et al., 2012). This provides a strong link between the innate and adaptive responses, because IFN-γ works on the activation

of the T-box transcription factor (T-bet) which is an important factor to T-cell development (Larosa and Orange, 2008).

1.2.4.1.3 T lymphocytes.

T-cells play a crucial role in the response to pathogens that takes place inside host tissues. They constitute 75-80% of the total lymphocytes in the blood (Tomasek et al., 2011). The development process for T-cells starts in the fetal liver during the period of early embryogenesis and later continues within a specialised primary lymphoid organ, the thymus (Swainson et al., 2013). T-cells are generated in the thymus; however, their precursors are found in the bone marrow. The cells in this stage do not mature fully and they do not express CD4 or CD8 receptors **(double negative T-cells)** (Murphy and Weaver, 2017, Gargani and Kitchen, 2012). T-cells pass several stages in the thymus to become mature cells with the ability to differentiate between self and non-self antigens. This process in the thymus will select the TCR that can bind to major histocompatibility complex (MHC) class I or class II proteins encoded by the MHC group of genes are found on the surfaces of cells that help the immune system recognise foreign substances. The MHC molecules are responsible for presenting antigens to T-cells (Murphy, 2012). The cells, which do not bind to the MHC-antigen complexes weakly, die by apoptosis so the rest of the cells will undergo **positive selection.** The positive selection indicates that the T-cell has the ability to interact with self-peptide-MHC complexes expressed by the cortical epithelial cells of the thymus (Figure1.2 A) (Harrington, 2019, Male et al., 2012).

In the same time, cells that respond to the MHC class II will keep the CD4 receptor and lose the type CD8 receptor so turn into T helper cells. The cells which respond to the MHC class I receptor will keep the CD8 receptor and lose the CD4 receptors and consequently become cytotoxic T-cells. After this stage, they will suffer the process of **negative selection**, in which the cells that bind strongly to the MHC/self-peptide will undergo apoptosis. This means that

an immature T-cell clone encoding a self-reactive T-cell is eliminated from the host (Figure 1.2 B) (Rhoades and Bell, 2009, Harrington, 2019). The cells are now ready to leave the thymus into the bloodstream. The product cells are considered to be immunologically naïve T-cells (Th0). Naïve T-cells are structurally mature but different from the activated or memory T-cells. That difference is because the effector and memory T-cells have previously encountered specific antigens (Punt, 2013, Berard and Tough, 2002). Naïve T-cells are characterized by the expression of L-selectin (CD62L), the absence of the markers of activation CD25, CD44 or CD69; and the absence of the memory CD45RO marker (De Rosa et al., 2001, Berard and Tough, 2002). T-cells are classified into two broad subtypes according to the structure of the T-cell receptor (TCR) molecules.

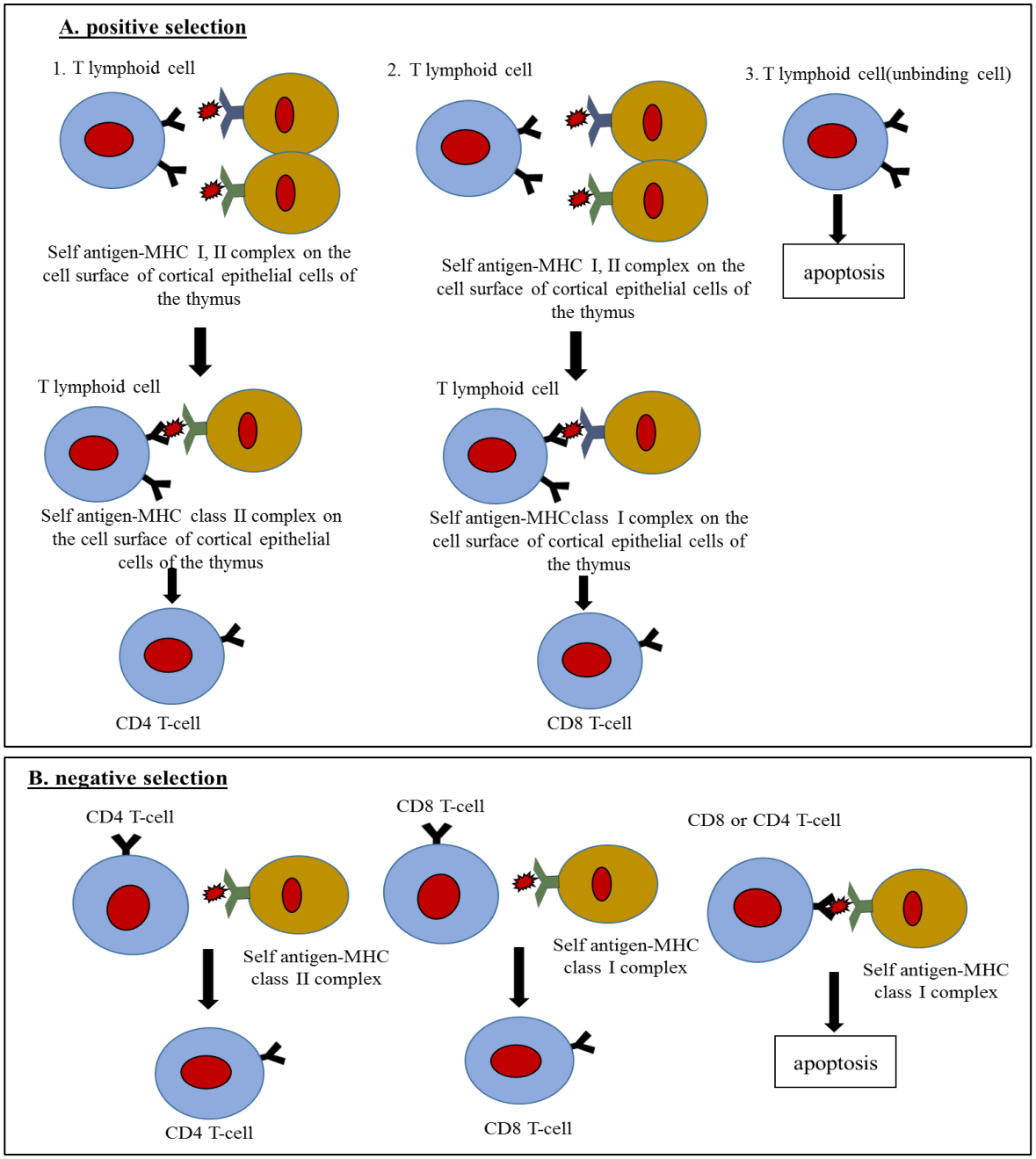

Figure 1-2 CD4 T-cell maturation in the thymus (positive and negative selection). A. positive selection for T lymphocytes bound weakly to self-peptide-MHC complexes. If a T-cell has the ability to bind to MHC class I, it will become a $CD8^+$ T-cell, while cells that bind to MHC class II will become $CD4^+$ T-cells. Apoptosis occurrs with the unbound cells. B. negative selection, cells that bind strongly to the self-peptide-MHC will undergo apoptosis. This means that an immature T-cell clone encoding a self-reactive T-cell is eliminated from the host. Only the T-cells that have not recognised the self-peptide will survive.

- **1.2.4.1.3.1 TCR-1 (γδ cells).**

TCR-1 (γδ cells) TCR surface membrane molecules are composed of γ and δ polypeptides.

These cells constitute 5% of the T-cells in general, and 10% of intraepithelial T-cells. They

are not limited to binding to antigens presented by the MHC molecules and their role in the immune response is less well defined compared to that of the αβ cells discussed below (Parham and Janeway, 2009, Hayday and Pao, 1998). The γδ T-cells recognize target cells in an MHC-independent model, harmonic with a lack of surface CD4/CD8 co-receptor expression (Davey et al., 2017). Functional and molecular analyses show that following infection with various pathogens, γδ T-cells respond earlier than αβ T-cells, also they emerge late, after the pathogen numbers start to decline, which means these cells may be involved in both establishing and coordinating inflammatory response (Chien et al., 2014). The γδ T-cells have been identified as the major initial producers of IL-17, and γδ T-cells are also early sources of IFN-γ and TNF-α (Chien et al., 2014, Cai et al., 2011, Stark et al., 2005, Zhao et al., 2018). The γδ T-cells activation model still is not fully defined (Chien et al., 2014).

- **1.2.4.1.3.2 TCR-2 (αβ cells).**

The TCR consists of α and β polypeptides and constitutes 95% of T-cells. They are sub-classified into two groups of cells: Th (T-helper cells) that are $CD4^+$ and Tc (cytotoxic T-cells) that are $CD8^+$. Th cells detect the antigens that are expressed on MHC class II, while the Tc cells detect antigens expressed on MHC class I (Murphy, 2012). Th cells can be classified more through subdivision into Th1, Th2, Th9, Th17, Th22, T follicular helper cells (Tfh) and T regulatory (Tregs). These cell populations are classified according to the cytokines they secrete (Figure 1.3) (Murphy, 2012, Eyerich et al., 2009, Brownlie and Zamoyska, 2013).

- **Th 1 cell.**

Th1 cells play an important role in the immune response against bacteria enclosed inside macrophages. Antigen uptake results in activation of macrophages and an increase in their intracellular killing capacity. Intracellular pathogens stimulate DCs and macrophages to produce cytokines favouring Th1 development, which are derived from an antigen-activated

$CD4^+$ T-cells, such as IFN-γ and IL-12. The prevalence of certain type of APCs plays a crucial role in developing specific types of Th cells and can influence whether a shift from the Th1 to the Th2 phenotype occurs. Macrophages express antigen most efficiently to Th1 cells and this cell specific expression will produce cytokines stimulating Th1 proliferation and increasing the dominance of this subset (Mak and Saunders, 2006). Th1 cells secrete IL-2, IL-3, IFN-γ, TNF-α, TNF-β and GM-CSF. Th1 cells play an important role in inflammation, cytotoxicity and delayed-type hypersensitivity (Murphy, 2012, Abbas et al., 2017).

- **Th2 cells:**

The infection with extracellular pathogens most often promotes the development of Th2 cells. Antigen-specific B cells play a more important role in antigen presentation to Th2 cells. IL-4 promotes Th2 proliferation and development (Mak and Saunders, 2006). Th2 cells secrete cytokines such as IL-4, IL-5, IL-6, IL-10, IL-13, TNF-β and GM-CSF when they are activated. Moreover, these cells support the activation of B-cells and encourage B-cells to generate different types of antibodies, such as IgE and some subclasses of IgG antibodies. Eyerich et al confirmed that Th2 cells can be found in the skin of patients with atopic eczema, psoriasis, or allergic contact dermatitis (ACD) (Eyerich et al., 2009). Thus, Th2 are also implicated in the pathogenesis of certain skin diseases (Duhen et al., 2009, Kagami et al., 2010).

- **Th17.**

In the 1990s the cytokine IL-17 was detected and new cloned forms of a $CD4^+$ T-cell was added to this family. The new member of helper T-cells was named Th17 cells (Tesmer et al., 2008, Sutton et al., 2009). When the DCs secrete IL-23 in response to antigens, IL-23 supports the conversion of $CD4^+$ T-cells to Th17 cells (Zhou et al., 2007). Moreover, it has been shown that IL-6, TGF-β and IL-1 are important cytokines in Th17 differentiation (Zhou

et al., 2007, Kurts, 2008). There are six subtypes of the IL-17 cytokines (A-F), however only IL-17A and IL-17F are secreted by Th17 cells (Wu et al., 2018). Th17 cells also produce other cytokines including IL-16, IL-17, IL-21, IL-22, IL-26 and TNF-α, in response to antigens (Eagar and Miller, 2008, Abul K Abbas, 2015, Shuttleworth et al., 2011). Th17 cells have been divided into two subtypes depending upon the cytokine milieu present during the differentiation process, pathogenic Th17 (that have been activated in the presence of IL-1β and IL-23) (Singh et al., 2013, Wu et al., 2018), and non-pathogenic Th17 cells (activated in the presence of TGF-β and IL-6) (Lee et al., 2012). Th17 cells participate in various autoimmune diseases such as systemic lupus erythematosus and the cytokine itself is defined as proinflammatory (Bettelli et al., 2007, Koshy et al., 2002, Kurts, 2008).

- **Th22**.

Th22 cells develop from naïve $CD4^+$ T-cells under specific conditions, which are determined by lineage-specific cytokines. Th22 cells are identified by the production of interleukin IL-22 (Jia and Wu, 2014). Naïve $CD4^+$ T-cells are converted to a Th22 cell phenotype in the presence of IL-6 and TNF-α, and this process is promoted by IL-1β (Duhen et al., 2009). Th22 cells secrete IL-22 and TNF-α, but not IFN-γ, IL-4, or IL-17 (Th1, Th2 and Th17 markers, respectively) (Eyerich et al., 2009, Jia and Wu, 2014). These cells have a profile that is clearly different from that of Th1, Th2, and Th17 cells (Eyerich et al., 2009). They express functional chemokine receptors that help them to reside in the skin. These include CCR4, CCR6, and CCR10 indicating their crucial roles in skin diseases (Duhen et al., 2009). Th22 cells are implicated in skin diseases such as psoriasis, atopic eczema, and allergic contact dermatitis. Moreover, Th22 cells are associated with different diseases, for example infections, autoimmune diseases, hepatitis and pancreatitis (Jia and Wu, 2014).

- **T follicular helper cells (Tfh).**

T follicular helper cells (Tfh) are specific subset of $CD4^+$ T-cells. These cells were first identified in the human tonsils (Breitfeld et al., 2000). Tfh cells are found in secondary lymphoid organs such as tonsils, spleen and lymph nodes, however, they can also be found in the blood circulation. Tfh cell differentiation is a very complex process that starts when DC prime naïve $CD4^+$ T-cells. Cytokines such as IL-6/IL-21 increase the ability for Tfh differentiation (Baumjohann et al., 2013).The transcription factor Bcl6 (B-Cell Lymphoma 6) is the master regulator for Tfh cell differentiation and development (Johnston et al., 2009, Nurieva et al., 2009, Crotty, 2014). Bcl6 induces CXCR5 molecules, which are a characteristic feature of Tfh cells (Choi et al., 2011). The chemokine receptor CXCR5 (B-cell follicle homing receptor) is a transmembrane protein that binds to the chemokine CXCL13 (Schaerli et al., 2000, Kim et al., 2001b, Fazilleau et al., 2009, Choi et al., 2011, Good et al., 2017). Tfh cells provide help to B-cells for germinal centre reactions. This reaction produces high-affinity antibodies through secretion of effector cytokines, such as IL-21 and IL-4, and through cell-to-cell interactions. Furthermore, Tfh cells support the generation of memory B-cells and long-lived plasma cells (Baumjohann et al., 2013, Crotty, 2011, King, 2009, McHeyzer-Williams et al., 2011, Jandl et al., 2017).

- **Th9**.

Th 9 cells are a group of $CD4^+$ helper T-cells produced after activation of naïve $CD4^+$ T-cells. The presence of TGF-beta and IL-4 are very important for this process. Th9 cells are characterised by secretion IL-9, IL-10, IL-21, CCL17, and CCL22, but not IL-4, IL-5, or IL-13 (Wilson and Wynn, 2009). Th9 cells play an important role in mucosal immunogenicity especially with allergic diseases such as asthma-induced allergic responses, airway inflammation, and allergic rhinitis through the secretion IL-9 (Neurath and Finotto, 2016). Th9 cells also play an important role in helminthic infections. They may produce harmful

effects due to development of chronic allergic inflammation and generaion of autoimmune disease (Kaplan et al., 2015, Matusiewicz et al., 2018).

- **Regulatory cells (Treg cells).**

Treg cells are known as $CD4^+$ T-cells with a liability to suppress potentially unwholesome activities of Th cells. Tregs cells are classified into two types; naturally occurring Treg (n Treg), which are generated in the thymus and induced Treg (I Treg) cells, which are created in the periphery through the action of TGFβ on naïve $CD4^+$ T-cells (Yadav et al., 2012, Hori et al., 2003, Fontenot et al., 2003). Both nTregs and iTregs work through inhibition of the other T-cells. They express the cell surface receptor CD25 which is also found in activated T-cells (Francisco et al., 2009). Treg cells are also characterised by the unique surface molecules such as CD62L and specific CD45 isoforms. They also express the Forkhead box P3 (Foxp3) transcription factor (Sakaguchi et al., 1995, Herbelin et al., 1998). Foxp3 is also called 'master regulator' or 'lineage-specification factor' which is responsible for the development of Treg cells (Hori et al., 2003, Fontenot et al., 2003). Tregs act either directly or indirectly to produce a regulatory and balanced environment, their action extends to various types of cells such as NK, NKT, B-cells, monocytes and dendritic cells. This includes the production of immunosuppressive cytokines (IL-10, TGF-β, IL-35), and interaction with costimulatory molecules on APCs (such as DCs) through cytotoxic T-lymphocytes associated protein-4 (CTLA4) (co-inhibitory molecule) (Thornton and Shevach, 1998, Schmidt et al., 2012).

- **Memory T-Cells.**

Immunological memory is a fundamental characteristic of adaptive immunity and the main aim of vaccination strategies (Chang et al., 2014). Naïve T-cells, which are $CD45RO^-$ when activated divide, multiply and express molecules especially cytokine proteins that will help in fighting pathogens. These cells have the ability to migrate to different tissues and convert

to memory T-cells. Long-lived memory cells are distinguished by expression of the characteristic receptor CD45RO (Murphy, 2012). Memory T-cells mainly aggregate in the memory T-cell pool (Kaech and Cui, 2012). IL-7 and IL-15 are important for memory T-cell survival (Surh and Sprent, 2008, Mazzucchelli and Durum, 2007, Raeber Miro et al., 2018). Memory T-cells are heterogeneous in phenotype and functional characteristics such as expression of surface-receptor molecules, location and trafficking properties (Willinger et al., 2005, Sallusto et al., 1999, Hamann et al., 1997). To survive memory T-cells are required to interact with self-peptides to maintain proliferation. Furthermore, memory T-cells are divided into two types; the central and the effector memory T-cells. Central memory T-cells express CCR7 and secrete IL-2, they remain in peripheral lymphoid tissues after re-stimulation. In contrast, effector memory T-cells settle down in tissues; they lack ability to express CCR7 but they express CCR3 and CCR5 (Murphy, 2012).

- **$CD8^+$ cytotoxic T lymphocytes ($CD8^+$ CTLs).**

$CD8^+$ CTLs react with antigens presented on MHC-I expressed on all nucleated cells. $CD8^+$ T-cells are also subdivided into Tc1, Tc2, Tc17, regulatory, and memory cells (Betts and Kemeny, 2009). This classification depends on patterns of cytokine production. $CD8^+$ CTLs are very specific towards a variety of antigens. Tc1 cells migrate to sites of inflammation and they are involved in viral clearance via general cytotoxic abilities and secretion of IFN-γ, IL-2, and TNF-β. While Tc2 cells are characterized by secretion of the cytokines IL-4, IL-5, IL-10, and TGF-β. The Tc2 cells are associated with other forms of pathologic conditions including various form of autoimmune disease (Burleson et al., 2015, Iezzi et al., 2006, Flaherty, 2012a). Tc17 cells produce IL-17 that participates in various conditions, such as infections, cancers, and autoimmune inflammation (Yen et al., 2009).

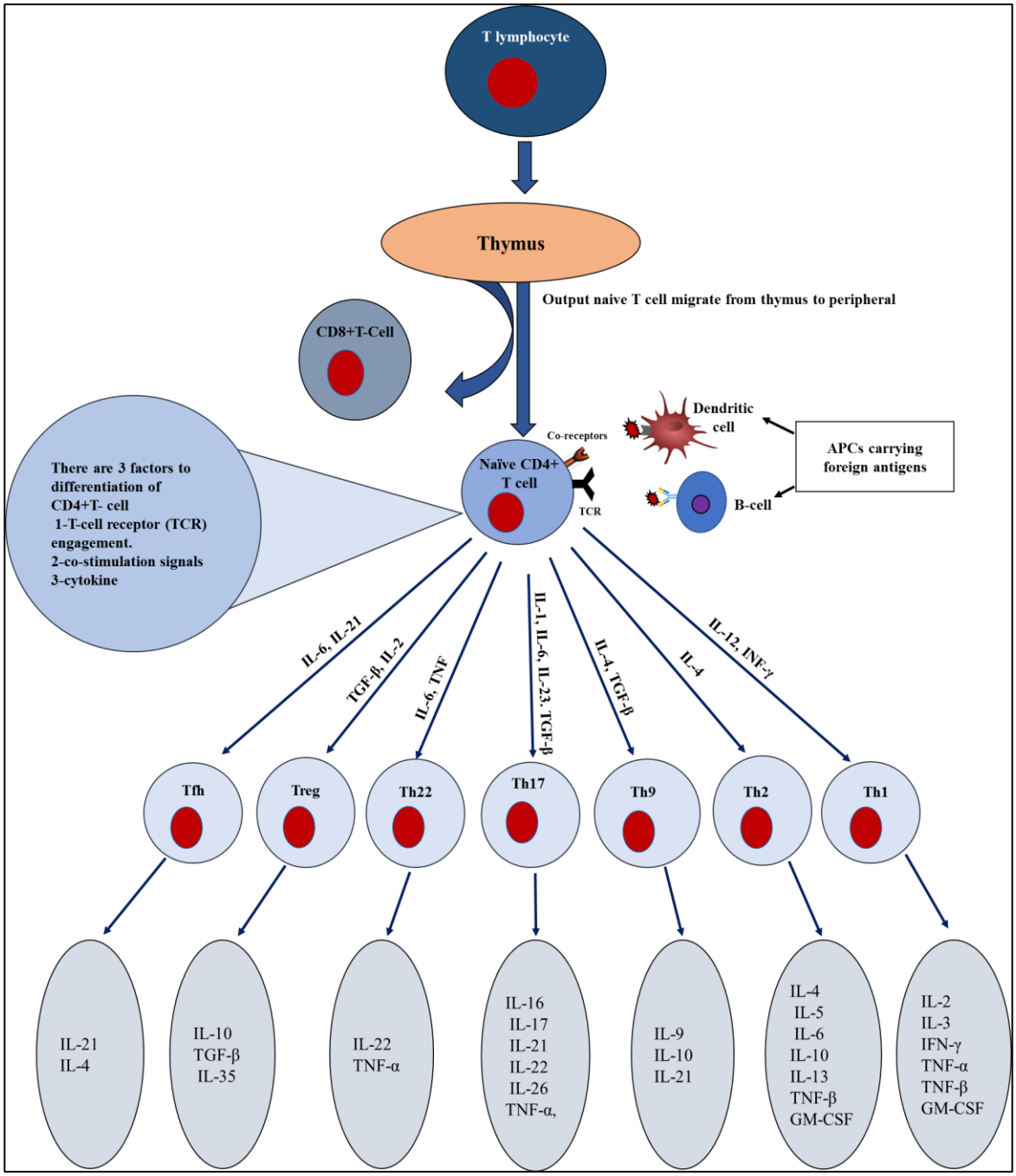

Figure 1-3 CD4⁺ T-cell differentiation. In the peripheral bloodstream when naïve cells encounter specific antigens, they will convert into several mature types of T-cell depending on stimulation signals and the cytokine environment. Each activated cell secretes specific types of cytokines.

1.2.4.2 Granulocytes.

1.2.4.2.1 Neutrophils.

Neutrophils (neutrophilic polymorphonuclear leukocytes) are one of the most important white blood cells in the human peripheral blood. They are characterised by a multi-lobed nucleus and neutrophilic granules. They exert an important function the engulfment and killing of extracellular pathogens (Frederick, 2000). Neutrophils are the most widespread leukocytes in the bloodstream. In normal conditions they constitute more than 50% of all bloodstream leukocytes. The percentage of neutrophils in the bloodstream will increase to 80% or more during bacterial infection (Shah et al., 2017). Neutrophils play an influential role in inflammatory reactions, and elimination of microorganisms and damaged cells. Furthermore, neutrophils perform an important role in the generation of inflammatory agents (chemotactic peptides, cytokines, leukotrienes and other lipid mediators) (Roos, 1998). Neutrophil cytoplasm is characterised by the granules which contain substances like myeloperoxidase, defensins, cathepsin-G and bactericidal/permeability-increasing protein, lactoferrin, cathelicidin and gelatinase. Neutrophils have the ability to regulate the function of monocytes and other lymphoid cells by secreting various types of cytokines such as IL-1β, IL-1ra, IL-8, TGF-β, and TNF-α (Khan, 2008).

1.2.4.2.2 Eosinophils.

Eosinophils are granulocytic leukocytes generated like other blood cells in bone marrow. They are important cells in innate immunity. They comprise 2% of the blood leukocytes in non-atopic individuals. Eosinophils participate in the pathogenesis of multiple inflammatory processes, including parasitic helminth infections and allergic diseases. Eosinophils also have phagocytotic ability and they kill ingested microorganisms (Rothenberg, 1998, Gleich and Loegering, 1984). They have granules that contain different types of enzymes and a toxic

protein. These granules are released during the activation of eosinophils (Logan et al., 2003). Eosinophils contribute to the inflammatory process by releasing a group of proinflammatory cytokines including TGF-α/β, IL-2, IL-4, IL-5, IL-10, IL-12, IL-13, IL-16 and IL-18, and they also produce chemokines such as RANTES and eotaxin-1 (Rothenberg and Hogan, 2006, Kita, 1996, Lim et al., 1995, Moller et al., 1996). Chemokines affect leukocytes through their specific selective receptors and are now known to attract material involved in leukocyte maturation, trafficking, and homing of cells. RANTES and eotaxin are very important chemoattractants for eosinophils. Eosinophils play a critical role in the process of allergic inflammation (Graziano et al., 1999, Khan, 2008).

1.2.4.2.3 Basophils and Mast cells.

Paul Ehrlich discovered and detected the basophils in 1879 based on their individual shape (Falcone et al., 2000). Basophils represent very small population of the white blood cells in the blood stream. They constitute 0.01- 0.3% of all leukocytes. They have large dark purple granules and a bilobed nucleus (Cawley and Hayhoe, 1973). They develop in bone marrow similar to mast cells but they generate from different precursor cells (Khan, 2008). Basophil maturation occurs in the bone marrow. They then circulate in the peripheral blood before moving to specific tissues. In contrast, mature mast cells take another path. They do not circulate in the blood but their maturation and differentiation processes occur in vascularised tissues (Galli, 1999). Basophils are non-phagocytic granulocytes. They exert their function by releasing pharmacologically active substances from their granules (Goldsby and Richard, 2003). Both mast cells and basophils release heparin. They also both release histamine and cytokines. They play an important role in mediating type I hypersensitivity reactions due to the IgE antibody that is attached to their cell membrane. The IgE antibody will stimulate the release of large quantities of mediators which yield local inflammatory process that result in

vascular and tissue reactions and the various allergic manifestations (Bain, 2017, Wedemeyer et al., 2000).

1.2.4.3 Antigen presenting cells.

1.2.4.3.1 Macrophages.

Macrophages are a family of mononuclear leukocytes. Macrophages are widely distributed throughout most tissues. Monocytes are differentiated into either tissue macrophages or DCs and the process is dependent on the local cytokine milieu and inflammatory stimuli (Yang et al., 2014, Tacke et al., 2007). Monocytes differentiate to macrophages in the intestine and the dermis during acute inflammation (Das et al., 2015). Macrophages are widely distributed throughout most tissues. The main role of the macrophage is the scavenging of dying cells, pathogens, and molecules through phagocytosis and endocytosis (Toews, 2009). They are large cells that differentiate from circulating monocytes after they enter tissues. There are several steps for generation and differentiation of macrophages. The interleukin-3 (IL-3), granulocyte-macrophage colony stimulating factor (GM-CSF), and macrophage colony stimulating factor (M-CSF) bind to specific receptors on progenitor cells to activate a sequence of differentiation steps essential in the monocyte development. Cytokines secreted from inflammatory cells during acute inflammatory responses play a vital role in regulating macrophage differentiation. Interleukin-1 (IL-1) and tumor necrosis factor (TNF-α) promote M-CSF and GM-CSF production (Kinne et al., 2009, Toews, 2009, Cole et al., 2014). Macrophages kill invading microorganisms by generating a respiratory burst and through activation of proteolytic enzymes. Furthermore, macrophages secrete a group of cytokines that attract leukocytes to the area of inflammation and trigger an acute inflammatory response. If macrophages receive inflammatory stimuli, they secrete cytokines such TNF, IL-1, IL-6, IL-8, and IL-12. Additionally, macrophages secrete chemokines, leukotrienes, prostaglandins, and complement. All of these molecules work together to induce increased

vascular permeability and recruitment of inflammatory cells (Arango Duque and Descoteaux, 2014). The most significant role of macrophages is that they act as antigen presenting cells; presenting antigens to T-cells. Thus, the macrophage is considered a bridge between the innate and adaptive immunity (Rhoades and Bell, 2013).

1.2.4.3.2 Dendritic cells.

Dendritic cells (DCs) are involved in both the priming of adaptive immune responses and induction of self-tolerance (Steinman, 1991, Steinman and Nussenzweig, 2002). They are distributed in small numbers in tissues that encounter various antigenic stimuli like the skin, the lining of the nose, the lungs and the stomach. DCs collect antigens from various tissues and transfer them to the lymph nodes. Following activation, the DCs initiate acquired immune responses through an interaction with the B and T-cells. The DCs can be classified into myeloid DCs and lymphoid DCs and they look similar to the plasma cells. These cells are also called plasmacytoid DCs (Murphy, 2012). Myeloid dendritic cells (MDCs) secrete IL-12 and are divided into two subsets; MDC1 and MDC2. MDC1 cells play a role in stimulating T-cells, while the MDC2 cells have a role in fighting wound infections. Lymphoid dendritic produce large amounts of IFN-α (Khan, 2008).

1.2.5 Antigen presentation and presenters.

The major histocompatibility complex (MHC) is a set of cell surface proteins essential for presenting antigens on the cell surface of APCs for recognition by T-cells. They perform this role through several biochemical modifications (Neefjes et al., 2011). The binding between TCRs and MHC molecules depends on the specific molecules on the T-cell surface known as cluster of differentiation (CD) $CD4^+$, and $CD8^+$. If the T-cell is a $CD4^+$ cell it will recognise antigens expressed on the MHC class II molecules, but if the T-cell is a $CD8^+$ cell, it will recognise antigens expressed on the MHC class I molecules. Furthermore, there are a

minority of T-cells that have gamma and delta receptors. They express alpha and beta chains, but they do not have CD4 or CD8. These cells require more than one signal to be activated (Dasgupta and Wahed, 2014). (See section 1.2.4.1.3.2).

1.2.5.1 MHC molecules structure. (Antigen presenters).

- **MHC class I molecule.**

MHC class I molecules are found on the all nucleated cells and express protein fragments of cytosolic and nuclear origin for cytotoxic $CD8^+$ T-cells. These antigens are degraded by cytosolic and nuclear proteasomes. MHC I is composed of a single polypeptide heavy α-chain of the binding platform which requires support by β-2-microglobulin protein. The heavy α-chain consists of 3 domains, α1, α2 and α3 (Figure 1.4 A). The long alpha chain possesses the entire peptide binding groove (Neefjes et al., 2011, Mosaad, 2015, Cresswell, 2012, Wieczorek et al., 2017, Coico and Sunshine, 2015). The long alpha chain of the MHC I is encoded by three genes on a specific region of chromosome 6, the genes are distinct but adjacent and are nominated A, B, and C genes. Moreover, β-2 microglobulin is encoded from a single gene on chromosome 15 (Figure 1.5) (Dasgupta and Wahed, 2014, Choo, 2007).) (See section 1.5.1). The position of HLA-A, B, and C genes are towards the telomere on chromosome 6 (Toews, 2009).

- **MHC class II molecule.**

The expression by the MHC class II molecules differs from class I, the MHC II is found on APCs only such as dendritic cells, macrophages, and B-cells. MHC II bound peptide antigens are recognised by $CD4^+$ T-cells. This molecule expresses peptides derived from the degradation of extracellular proteins after the APCs have internalised them. The MHC class II molecule consist of two separate polypeptide chains of equivalent length. These two chains known as α and β dimerise, and when they bind together form a peptide binding groove, α1

pairs with β1 on the exterior surface α2 pairs with β2 on the interior surface(Choo, 2007, Wieczorek et al., 2017, Coico and Sunshine, 2015). Furthermore, MHC II differs from MHC I, because both α and β chains have cytoplasmic tails (Figure 1.4 B) (Coico and Sunshine, 2015, Choo, 2007). The map of MHC genes is constantly being revised as new information is added (see figure 1.5). The MHC II is encoded by three distinct but adjacent genetic clusters on the specific region of chromosome 6. These groups of genes are called DP, DQ, and DR (Figure 1.5) (Coico and Sunshine, 2015). (See section 1.5.1). The position of HLA-D is towards the centromere. In sequence, they are separated from the class I genes by the complement protein genes. Each part of these regions is known to contain multiple genes (Toews, 2009).

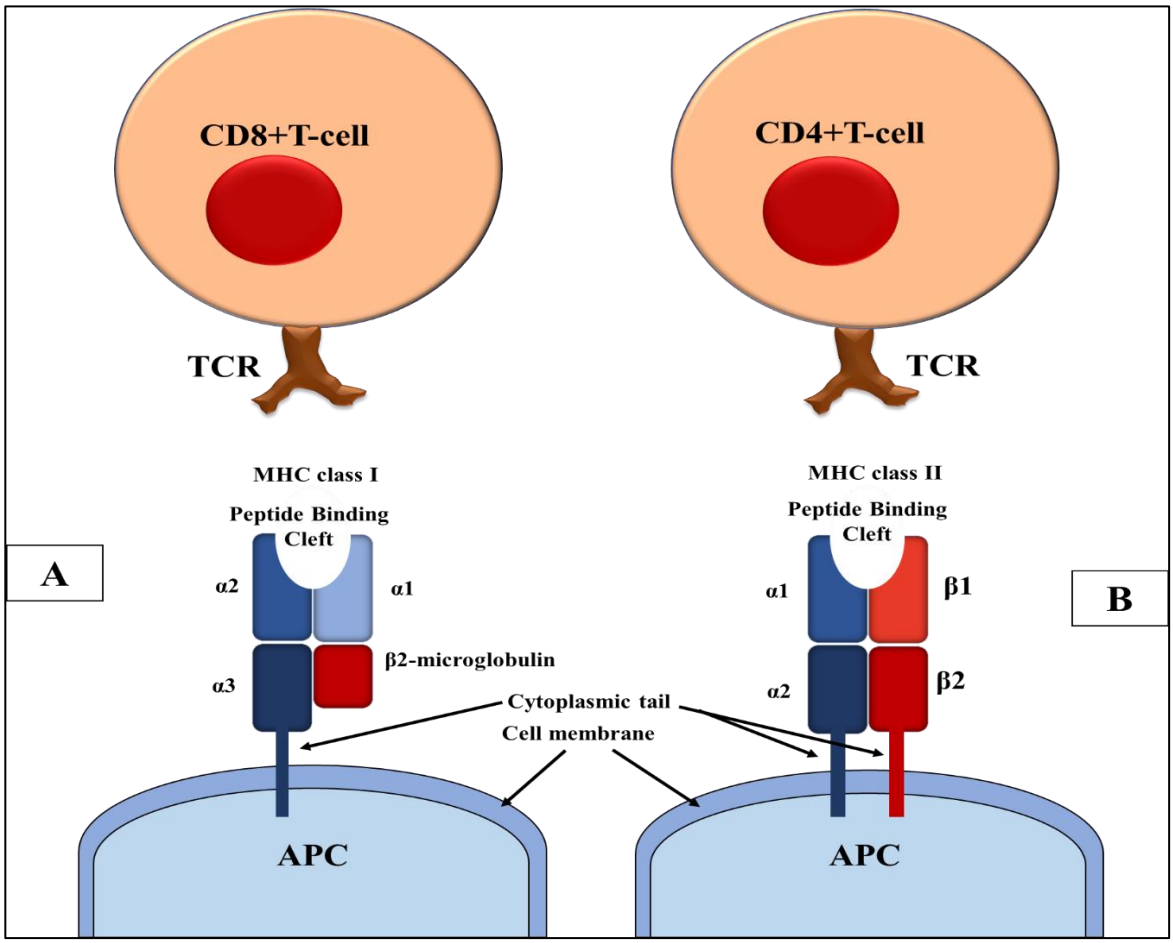

Figure 1-4 Figure MHC class I and II structures. A. MHC class I has heavy α-chain consisting of 3 domains, α1, α2 and α3. These bind with Beta-2 microglobulin and this expresses one cytoplasmic tail. MHC I presents antigen to $CD8^+$ T-cells. B. MHC class II consists of two separate polypeptide chains of equivalent length. α and β chains dimerise; α1 pairs with β1 on the exterior, and α2 pairs with β2 on the interior. This class expresses two cytoplasmic tails. MHC II presents the antigens to $CD4^+$ T-cells.

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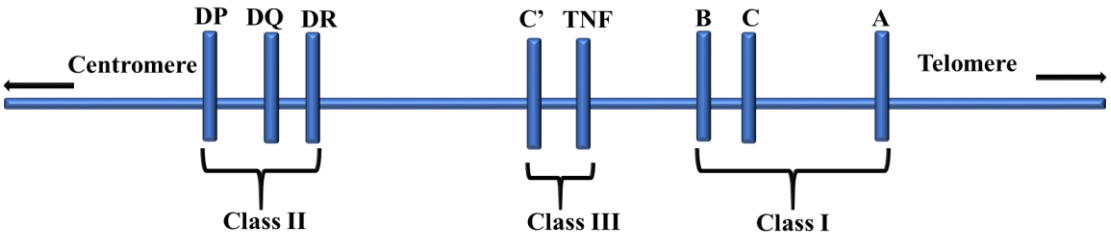


Figure 1-5 Human HLA on the chromosome 6, MHC II is encoded by three distinct genetic clusters HLA-DR, DP, and DQ. MHC class I is encoded by A, B and C genes. Class III cluster of genes is encoded by TNF and C' (complement genes), which is primarily influential in inflammatory factors, but it is not involved in antigen presentation.

1.2.5.2 MHC class I antigen presentation.

The MHC class I molecules express peptide fragments of cytosolic and nuclear (intracellular) proteins such as viral proteins, at the cell surface. These intracellular proteins are degraded by cytosolic and nuclear proteasomes. The derived peptides are translocated into the endoplasmic reticulum (ER) by carrier or transporter associated with antigen presentation (TAP) to the MHC class I molecules. In the ER, the MHC class I molecules are not stable until they bind to peptides deep in the MHC class I peptide-binding groove. The binding groove mainly accommodates peptides of 8-9 amino acids (Neefjes et al., 2011, Mosaad, 2015, Cresswell, 2012). MHC class I-peptide complexes will liberate from the ER and then the MHC I-peptide complex traffics through the Golgi apparatus to the plasma membrane to express the processed peptide on the cell surface for $CD8^+$ T-cells (Figure 1.6) (Neefjes et al., 2011, Zarling et al., 2003, Hewitt, 2003).

1.2.5.2 MHC class II antigen presentation.

Peptide presentation by MHC class II molecules differs from MHC class I, because of the site and mechanism of processing. MHC class II binding only occurs in professional APCs, such as DCs, macrophages and B cells. The α- and β-chains of MHC class II are composed in the ER, and confederate with the invariant chain (Ii). This complex called Ii-MHC class II complex, leaves the ER, travels through the Golgi apparatus, where it will be transported to a late endosomal compartment. In this loci, Ii is digested due to the environment which is proteases rich and acidic. After digestion of Ii peptide, the remaining structure is MHC class II-CLIP. CLIP binds to a specific place in the peptide-binding groove of the MHC class II heterodimer. MHC class II molecules utilise HLA-DM to simplify the exchange of the CLIP fragment to a specific peptide derived from a protein degraded in endosomes or lysosomes. The MHC class II molecules then move to the plasma membrane to present their peptide cargo to $CD4^+$ T-cells (Figure 1.7) (Denzin Lisa et al., 2005, Neefjes et al., 2011, Guermonprez et al., 2002).

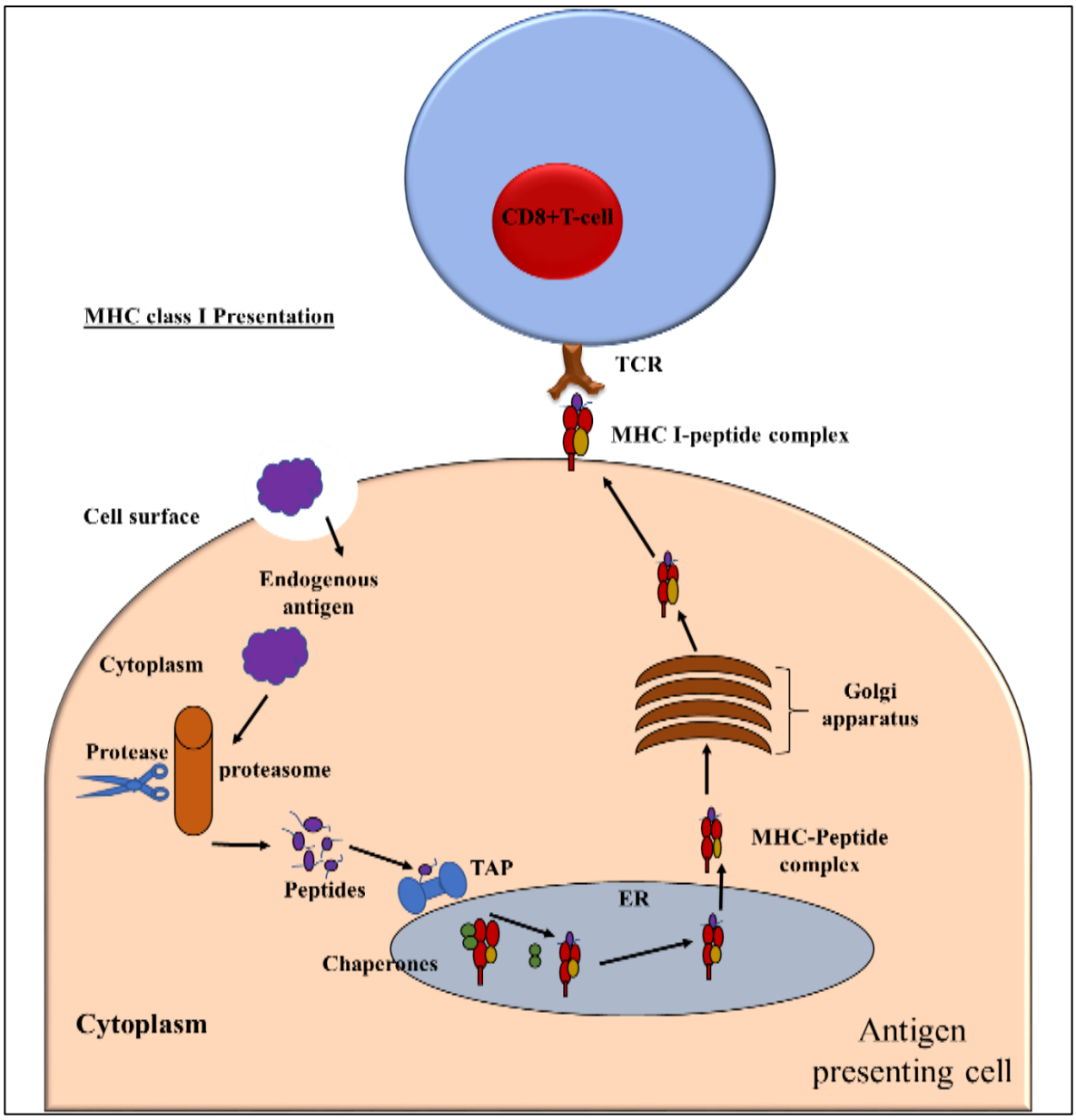


Figure 1-6 MHC class I presentation. The endogenous peptides enter the proteasome and are degraded by proteases. They are derived from the ER by the Transporter associated with Antigen presenter (TAP), where they bind to MHC I. This complex then moves to the Golgi apparatus and finally to the cytoplasmic membrane to encounter $CD8^{+}$T-cells.

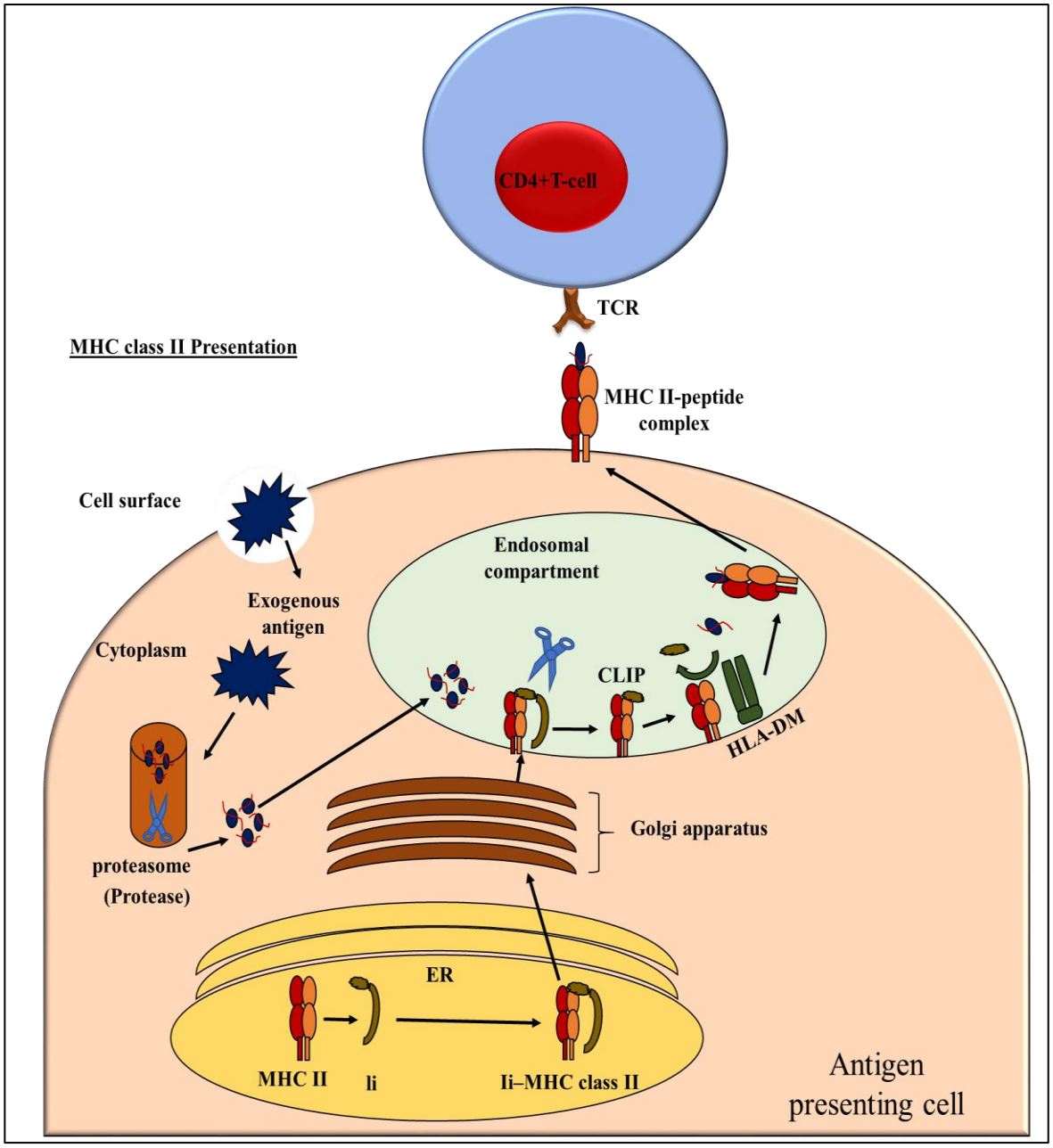


Figure 1-7 MHC class II presentation. The exogenous peptides enter the cell and are degraded by protease enzymes. They then move to bind with MHC II in the endosomal compartment, where the exchange of the CLIP fragment with a specific peptide occurs, with the help of HLA-DM. Then, the MHC II-peptide complex moves to the cytoplasmic membrane to express the antigen to $CD4^+$ T-cells.

1.2.6 The role of the T-cell receptor (TCR) in mediating an immune response.

1.2.6.1 TCR structure and diversity.

The T-cell receptor (TCR) is a dimeric (αβ or γδ) and highly variable membrane protein existing on the surface of the T-cells. It is responsible for the discrimination of the antigen-major histocompatibility complexes. A binding interaction may trigger an immune response (Lee, 2016, Stauss and Xue, 2014, Bassing et al., 2002). Each chain of the TCR consists of two extracellular domains: a Variable (V) region and a Constant (C) region. These two parts belong to the immunoglobulin superfamily. A transmembrane/cell membrane-spanning region binds to the constant region and expresses short cytoplasmic tail at the C-terminal end (Figure 1.8). The pairs of α/β or γ/δ evolve from the somatic DNA recombinations of germline gene segments through T-cell development. TCR genes for α and δ are on chromosome 14 while β chain and γ chain gene loci are on chromosome 7 (Boehm and Rabbitts, 1989). The capability of the TCR to bind with an enormous number of antigens both foreign and self is due to the antigen recognition site (or variable region). The region is created by a unique process of somatic recombination. The presence of a pool of multiple V(D)J gene segments in the DNA produces a highly diverse TCR composition. Moreover, the addition or removal of genes is important for T-cell antigen recognition (Setton et al., 2016, Lee, 2016, Freeman et al., 2009). Site-specific V(D)J recombination subgenic elements are classified as V (Variable), D (diversity), and J (joining) families (Schwarz and Bartram, 1996). Genes encoded for variable domain of both the TCR α-chain and β-chain are characterised by three hypervariable or complementarity regions (CDRs); (CDR1, 2, and 3). CDR3 is the main CDR in charge of recognising the peptide antigen. However, CDR1 of the alpha chain has also been shown to interact with the N-terminal site of the antigenic

peptide, while, the same CDR1 of the beta chain interacts with the C-terminal part of the peptide. CDR2 is believed to interact with the MHC molecule (Figure 1.8) (Stauss and Xue, 2014). TCRs are very similar to immunoglobulins in many aspects, but the TCRs are stably expressed on the cell surface and are never secreted (David et al., 2012).

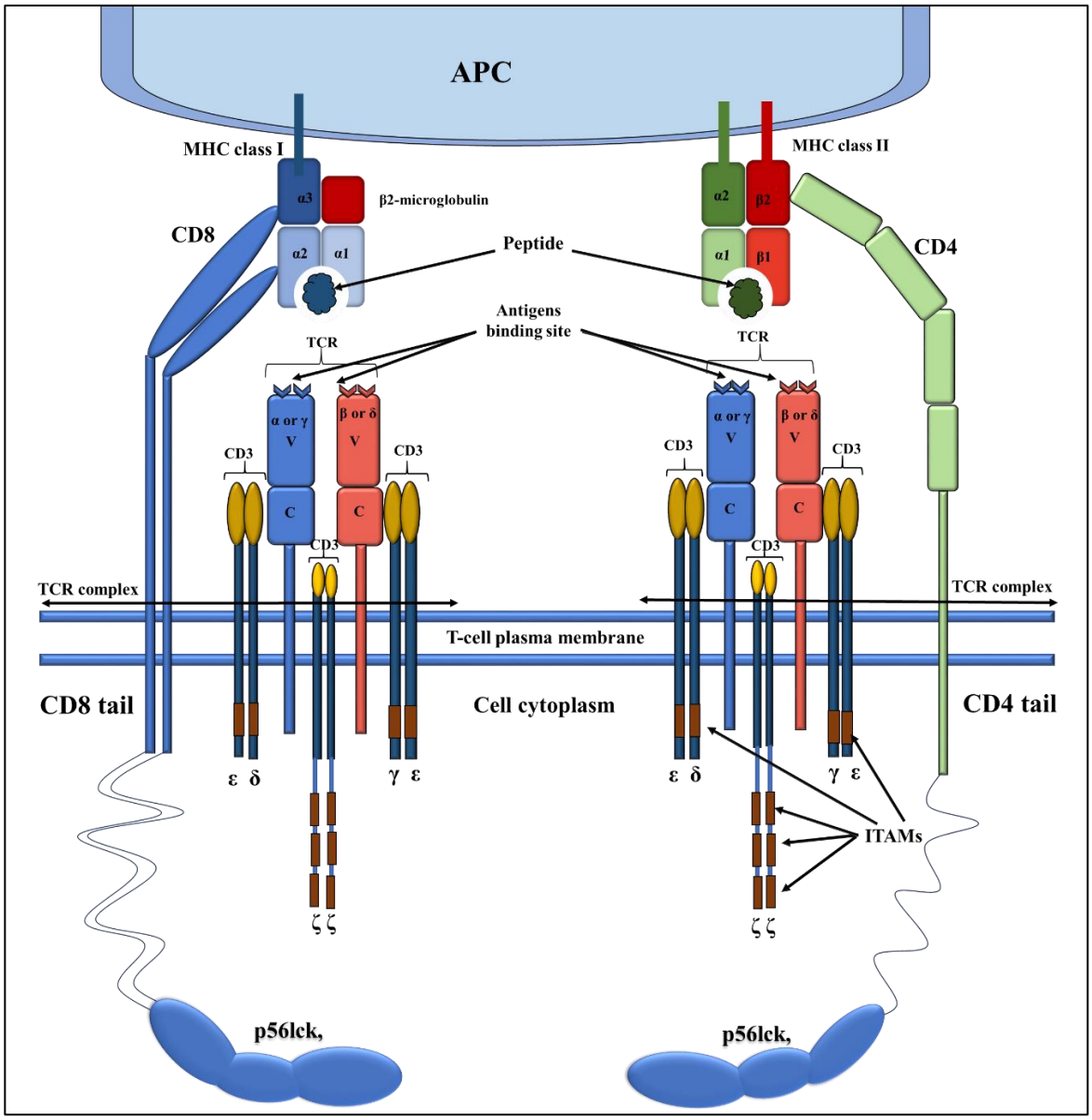


Figure 1-8 T-cells receptor (TCR) with CD3 and CD4/CD8 structures. The T-cell receptor consists of α and β chains in αβ T-cells, and it is composed of γ and δ chains in γδ T-cells. Antigen binding site is at the end of the variable region, which has the complementarity determining regions (CDRs). The CD3 receptor is composed of two couples of chains ε and γ, ε and δ. Further, there are two ζ chains. CD4 molecule in $CD4^+$ T-cell (responsible for recognising the antigens on MHC II) and CD8 molecules in $CD8^+$ T-cell (responsible for recognising the antigens on MHC I) are attached to p56lck, which is a tyrosine kinase and it participates in T-cell signalling.

1.2.6.2 The CD3 receptor complex.

The T-cell receptor-CD3 complex (TCR-CD3) play an essential role in the differentiation, survival, and function of T-cells. The CD3 complex of polypeptides is expressed on the T-cell surface (Call et al., 2002). CD3 is composed of six different chains responsible for ligand recognition, (Ley et al., 1989). These chains form two heterodimers CD3γε, CD3δε, as well as the CD3ζζ homodimers (Figure 1.8) (Bettini et al., 2017). A T-cell response to foreign antigen is initiated by the binding of the T-cell receptor (TCR)-CD3 complex to a foreign peptide express to an MHC molecule on an antigen-presenting cell. The signal deriving from this binding is transmitted into the cytosol to stimulate many signalling proteins. The question here is how the signals are transferred from outside the cell to inside? The cytoplasmic tail of the CD3 complex contains an immunoreceptor tyrosine-based activation motif; CD3ε, CDδ, and CD3γ each contain one immunoreceptor tyrosine-based activation motif (ITAM) and that of CD3ζ contains three ITAMs, as shawn in figure 1.8. Therefore, TCR-CD3 complex comprises 10 ITAMs. The binding betweenTCR-CD3 complex and an antigen leads to phosphorylation of the ITAM residues, this process will produce recruitment and activation of multiple downstream signalling molecules including enzymes and adaptor proteins (Ngoenkam et al., 2017).

1.2.6.3 Stabilizing molecules.

The TCR binds to the MHC molecules with low affinity. Therefore, additional molecules are necessary to support the complex. CD4 and CD8 both play a role in stabilizing the T-cell interaction the APCs. These stabilising molecules have a transmembrane region which binds to APCs and a cytoplasmic tail. CD4 binds to the MHC class II molecule and CD8 to the MHC class I molecule. The tail of CD4 and CD8 plays a role in signal transduction also to the nucleus by phosphorylation of serine residues in the cytoplasmic tail. This process will trigger p56lck kinase activation (Figure1.8) (Flaherty, 2012b, Artyomov et al., 2010). CD4

is a single-chain consisting of four immunoglobulin-like domains (D1, D2, D3, and D4) (Figure 1.8). The binding between CD4 and MHC II molecules occurs through the lateral face of the first domain, D1 binds to a site on the β2 domain of the MHC class II molecule. This connection is away from the site where the T-cell receptor binds to MHC-peptide complex. The binding of the CD4 molecule to the T-cell receptor enhances the transfer of signalling complexes. CD4 interacts strongly with a cytoplasmic tyrosine kinase called Lck. The tyrosine kinase Lck molecule associates most strongly to the cytoplasmic tails of CD4 and CD8 coreceptors. Lck plays crucial role in signal transduction and activation of the T-cell receptor. When CD4 molecule and the T-cell receptor simultaneously bind to the same MHC class II-peptide complex, T-cell sensitivity to this antigen is increased and T-cells could requires 100-fold less antigen for activation. The CD8 molecule consists of an α and a β chain, and each one contains a single immunoglobulin-like domain connecting to the cell membrane by a segment of extended polypeptide chain. CD8 binds to the α3 domain of an MHC class I molecule (Figure 1.8). CD8α and β chains interact with α2 and α3 domain of the MHC class I molecule. Similar to CD4, CD8 also binds Lck through the cytoplasmic tail, resulting in the same action. Furthermore, CD8 participate in the response to antigen increases the sensitivity of T-cells to antigen presented by MHC class I molecules by about 100-fold (Janeway et al., 2001).

1.2.6.4 Co-stimulatory and co-inhibitory molecules.

There are two signals needed for T-cell activation. The first signal for activation is formation of TCR MHC-peptide complex (Signal 1). This signal is not enough to stimulate a full immune response. There are co-stimulatory signals which occur through co-stimulatory receptors and their symmetric ligands on APCs (signal 2) (Mueller et al., 1989, Zhang and Vignali, 2016, Lafferty and Cunningham, 1975). For example, the more distinguished co-stimulatory pathways are the interaction between the co-stimulatory receptor CD28 (on the

T-cells) with its ligands B7.1 (CD80) and B7.2 (CD86) on the APC. The CD28:B7 axis produces a strong stimulatory signal for TRC activation and IL2 production will lead to an increase the T-cell proliferation. To prevent an immune response, there is another mechanism dependent on co-inhibitory receptors, which dampens co-stimulatory signals (Zhang and Vignali, 2016). B7.1 also works as a co-inhibitory receptor. If this molecule interacts with the co-inhibitory receptor CTLA4 (CD152) on the T-cells it can prevent T-cell activation. Other important co-inhibitory receptors are listed in Table 1.1, Figure 1.9 (Zhang and Vignali, 2016, Goronzy and Weyand, 2008, Naisbitt et al., 2000).

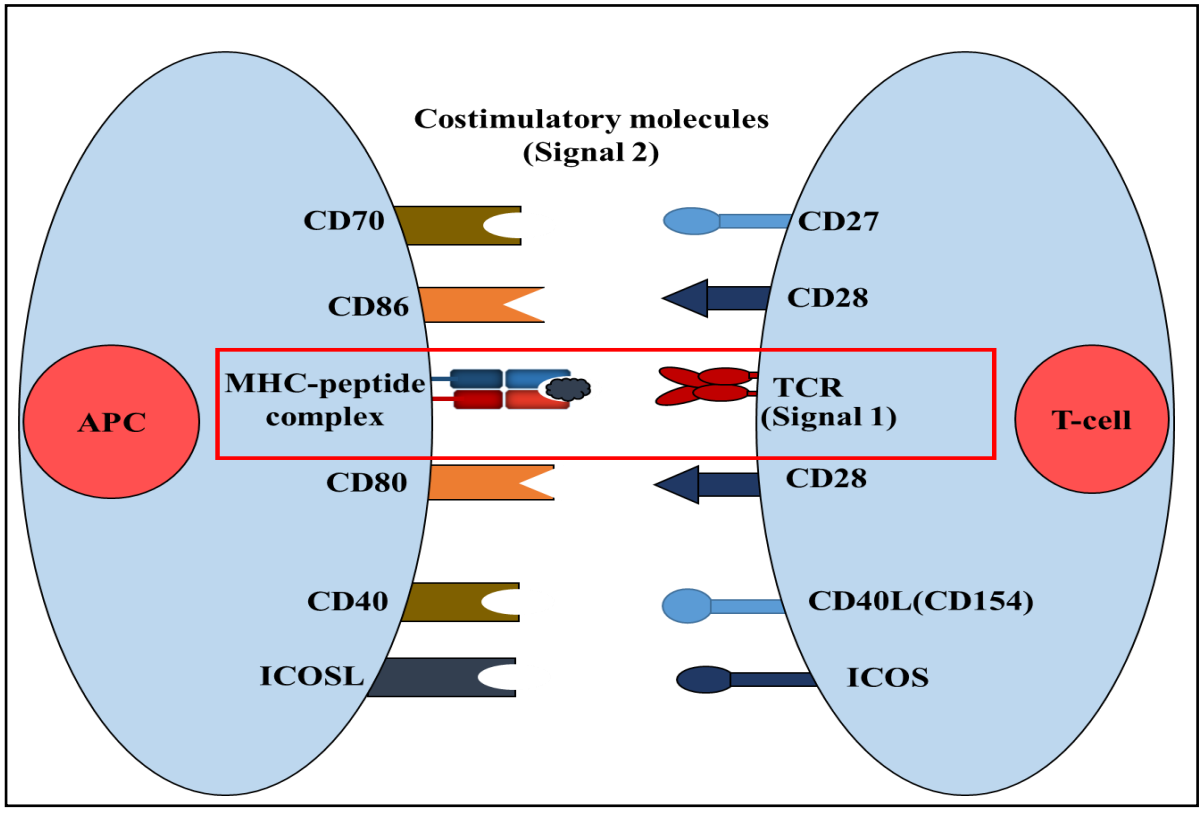


Figure 1-9 Co-stimulatory molecules. T-cell activation is triggered by a determined MHC-peptide complex presented by APCs to the TCR complex and T-cell costimulatory signals provided by the main CD28 interactions with CD80 and CD86. There are many costimulatory molecules involved in the interaction. The red box represent signal 1 and the binding among the co-stimulatory molecules between T-cell and the APC represent signal 2.

Table 1-1 Co-stimulatory and co-inhibitory molecules. These are binding interaction between T-cells and antigen-presenting.

Co-stimulation molecules		Co-inhibition molecules	
T-cell	Antigen-presenting cell	T-cell	Antigen-presenting cell
CD40-L	CD4	PD-1	PD1-L
ICOS	ICOS-L	CTLA-4	CD80/CD86
CD28	CD80/CD86	BTLA	HVEM
CD27	CD70		

1.2.6.5 Cytokines.

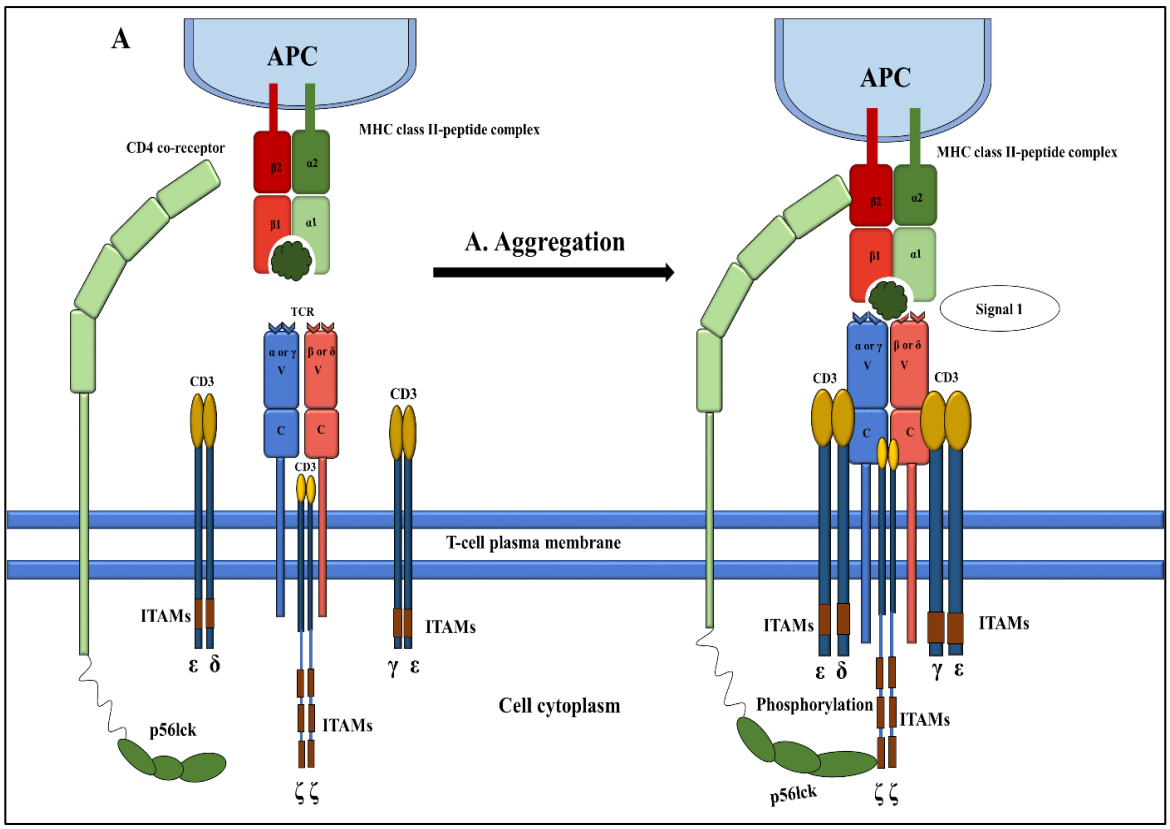
Cytokines are small polypeptides secreted by cells that have a specific influence on the interaction and communication between cells. The name cytokine is given to several groups of secretory proteins including lymphokines (cytokines secreted by lymphocytes), monokine (cytokines secreted by monocytes), chemokines (cytokines with chemotactic activities which attract other cells) and interleukins (this type of cytokine is made by one leukocyte and effects other leukocytes) (Zhang and An, 2007). Cytokines play a fundamental role in cell signalling, which means the communication within the immune system and they provide the immune system and host cells a mechanism to interchange information. Cytokines perform their role by binding to the particular receptors that deliver signals to recipient cells (McInnes, 2017). Cytokines may affect the same cells that secrete them (autocrine action), or act on nearby cells (paracrine action). Furthermore, some cytokines act on distant cells and tissue (endocrine action) (Zhang and An, 2007). Naive T-cell differentiation is promoted by cytokines, and their actions are controlled by various types of cytokines. Th1 cells produce interferon (IFN-γ) and interleukin (IL-2), and their differentiation is supported by IL-12 and IFN-γ. Th2 cells produce IL-4, IL-5, IL-9, IL-10, and IL-13, and their differentiation is supported by IL-4 (Eagar and Miller, 2008). This has been discussed in greater detail in section 1.2.4.1.3.

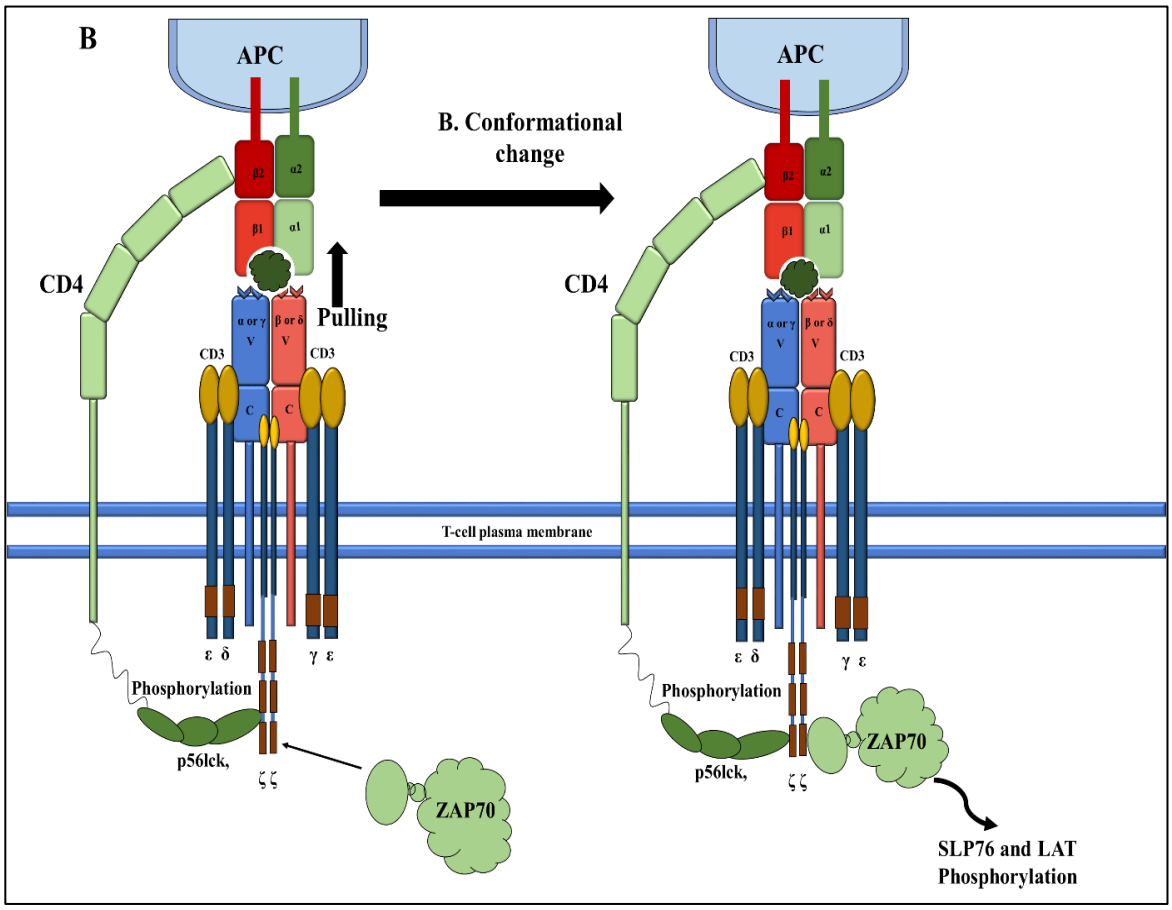
1.2.6.6 TCRs in drug hypersensitivity

- **TCR signalling.**

The interaction between TCR and a peptide-loaded major histocompatibility complex (pMHC), produces responses that differ in severity and duration (Varma, 2008). TCR-peptide-MHC complexes will trigger the TCR and transduce signals across the plasma membrane (Choudhuri and van der Merwe, 2007, Kuhns et al., 2006). These signalling pathways mediate T-cell proliferation, differentiation and cytokine secretion (Choudhuri et al., 2005). TCR triggering has been classified into three categories **aggregation, conformational change, and segregation,** to initiate signal transduction (Figure 1-10) (Choudhuri et al., 2005, van der Merwe and Dushek, 2011, Choudhuri and van der Merwe, 2007). TCR signalling is enriched by $CD4^+$ and $CD8^+$co-receptors via a mechanism called co-receptor heterodimerization. They bind to the peptide-MHC TCR complex (Locksley et al., 1993, Schilham et al., 1993, Choudhuri et al., 2005). The TCR-CD3 complexes can aggregate following TCR clashes with peptide-MHC complexes. This will promote phosphorylation processes. This **aggregation** could, increase the, closeness of associated Lck molecules (Figure 1.10 A) (Cooper and Qian, 2008). In the **conformational change model** has been proposed for triggering TCR at very low densities of agonist peptide-MHC molecules (van der Merwe and Dushek, 2011). This model of TCR triggering suggests that the TCR undergoes a conformational change during T-cell activation of lipid vesicles appear mimicking the lipid structure of the plasma membrane. This lipid vesicles promote the folding of the CD3ζ chain into a state where it is unable to be phosphorylated by Lck (Aivazian and Stern, 2000). After TCR-CD3 phosphorylation by Lck this process will produce a binding place for the action of another tyrosine kinase called ZAP-70 (zeta chain associated protein of 70 kDa) (Smith-Garvin et al., 2009). Sequentially ZAP-70 phosphorylates another two molecules called SLP76 (SH2-domain containing leukocyte

protein of 76kDa) and LAT (linker for T-cells activation). These molecules work together to trigger Ca_2^+ and diacylglycerol (DAG) release, which leads to T-cell activation (Figure 1.10 B) (Li and Rudensky, 2016). The final triggering model is called **segregation.** This model suggests that there is a redistribution of the TCR-CD3 complex with respect to other cell membrane-associated proteins. New molecules such as CD45 prevent Lck from constitutively proceeding with the TCR signalling cascade (Figure 1.10 C) (van der Merwe and Dushek, 2011, Nika et al., 2010).


A
APC
MHC class II-peptide complex
CD4 co-receptor
β2
α2
β1
α1
A. Aggregation
TCR
α or γ V
β or δ V
C
CD3
CD3
CD3
T-cell plasma membrane
ITAMs
ε δ
ITAMs
γ ε
Cell cytoplasm
p56lck
ITAMs
ζ ζ
APC
MHC class II-peptide complex
Signal 1
CD3
CD3
ITAMs
ITAMs
ε δ
γ ε
Phosphorylation
ITAMs
p56lck
ζ ζ


B
APC
B. Conformational change
CD4
Pulling
α or γ V
β or δ V
CD3
CD3
C
T-cell plasma membrane
ε δ
γ ε
Phosphorylation
p56lck,
ζ ζ
ZAP70
APC
CD4
Phosphorylation
p56lck,
ZAP70
SLP76 and LAT Phosphorylation

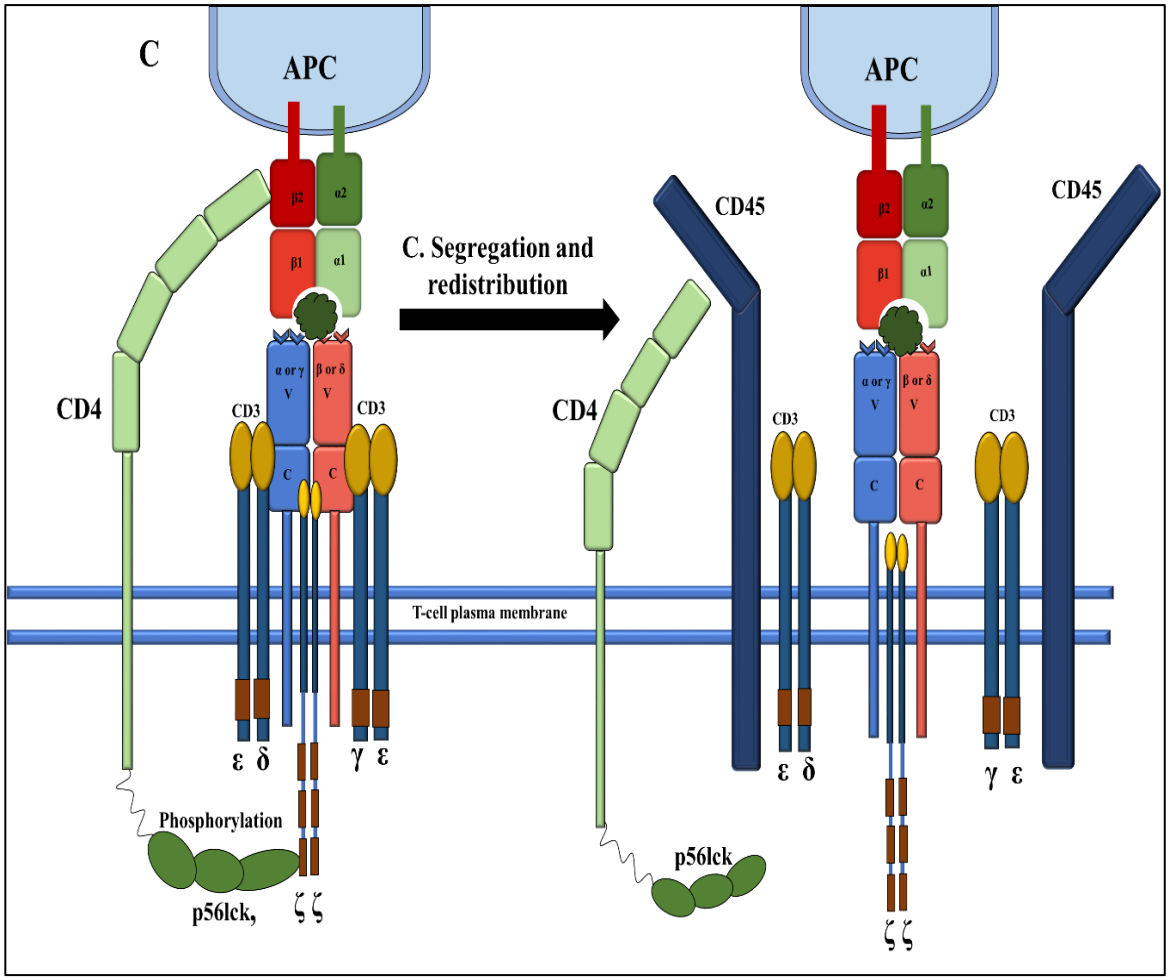


Figure 1-10 T-cell signalling A. binding T-cell to peptide-MHC complex (Signal 1), TCR-CD3 will aggregate together. The co-receptor will bind to the peptide–MHC complex as the T-cell receptor (TCR) brings co-receptor-associated LCK into proximity with TCR-CD3 immunoreceptor tyrosine-based activation motifs (ITAMs). B. conformational change, when zap70 become active and produces phosphorylation for SLP76 and LAT, these molecules work to trigger Ca_2^+ and diacylglycerol (DAG) release that will lead to serial events of T-cell activation. C. Segregation, the TRC-CD3 complex will redistribute by CD45 molecules. (A and B figures in the previous page).

1.3 Adverse Drug Reaction (ADR).

An adverse drug reaction (ADR) is defined as a response to a drug that is noxious, unintended or undesired, occurring at doses normally used for diagnosis or treatment of disease (World Health Organization, 1966, Edwards and Aronson, 2000). Although this definition has been used for a long period of time, several adaptations have been suggested. This is because some drugs are noxious but not really harmful, or just produce some irritation that combines with the treatment. For example, a persistent cough in patients using angiotensin-converting enzyme (ACE) inhibitors to treat hypertension and congestive heart failure (Cialdai et al., 2010). An adverse drug event (ADE) occurs in patients during or after the treatment, as a result of the exposure to the drug but it is not necessarily caused by the drug itself or from the mechanism of drug action (Asscher et al., 1995, Pirmohamed et al., 1998). ADRs are always adverse events but not all ADEs are ADRs. Finney was first to describe and distinguish the difference between the two terms (ADRs and ADEs) (Finney, 2006). Adverse drug events produce a significant patient morbidity and mortality; they are still underappreciated and sometimes not fully understood (Nebeker et al., 2004).

1.3.1 Adverse drug reactions, health burden and epidemiology.

ADRs are undesirable drug effects that have considerable economic as well as clinical costs. These reactions lead to hospital admission, prolongation of hospital stay and emergency department visits. Pharmacovigilance, which is defined as the science and activities relating to the detection, assessment, understanding and prevention of adverse effects or any other drug-related problem. ADRs are known to result in a high morbidity and mortality (Sultana et al., 2013, Formica et al., 2018). The burden on the health services and hospitals as a result of an ADRs is very high. ADRs are associated with losses in human life, reduced financial expenses and annual resources. Drug-related hospitalisation, and the output losses, are related to the nature of the ADRs (Roughead, 2002, Pirmohamed et al., 2004). The annual

cost of ADRs in Canada is estimated to be about $35.7 million (Wu et al., 2012). Furthermore, in the Children's Hospital of Michigan the total Intensive Care Unit (ICU) cost was increased by 3.5 fold, and length of hospitalisation was increased by 3.8 fold due to various cases of ADR (Du et al., 2013). In Europe, it has been estimated that approximately 5 % of all hospital admissions are caused by ADRs, and that ADRs cause 197,000 deaths per year throughout Europe (Bouvy et al., 2015). A prospective study in the UK estimated that of 18 820 patients who were admitted to hospital over a period of 6 months, 6.5% of admissions were due to ADRs. Further, the fatality rate was 0.15% and the annual cost to the National Health Service (NHS) was estimated at £466 million (Pirmohamed et al., 2004). Following all these studies, it has become extremely difficult to ignore the problem of ADRs and their effect on the health care system and human health (Bharadwaj et al., 2012).

1.3.2 Drug Allergy.

First use of the term "allergy" was in 1906 by Clemens von Pirquet to describe the reaction that occurs on second exposure to a substance termed as an "allergen" *(*foreign substance) (Shulman, 2017). The definition of allergy was updated by EAACI in 2001 and the World Allergy Organization (WAO) also advocated this term in 2003. Furthermore, they proposed the general definition as "a hypersensitivity reaction initiated by specific immunological mechanisms" (Johansson et al., 2004). Drugs can activate a spectrum of immunologically-mediated hypersensitivity reactions via various mechanisms (Warrington et al., 2018).

1.3.3 Classification of ADRs.

There are two commonly used classification systems for ADRs. The original classification was known as the type A and type B classification (Figure 1.11) (Patton and Borshoff, 2018).

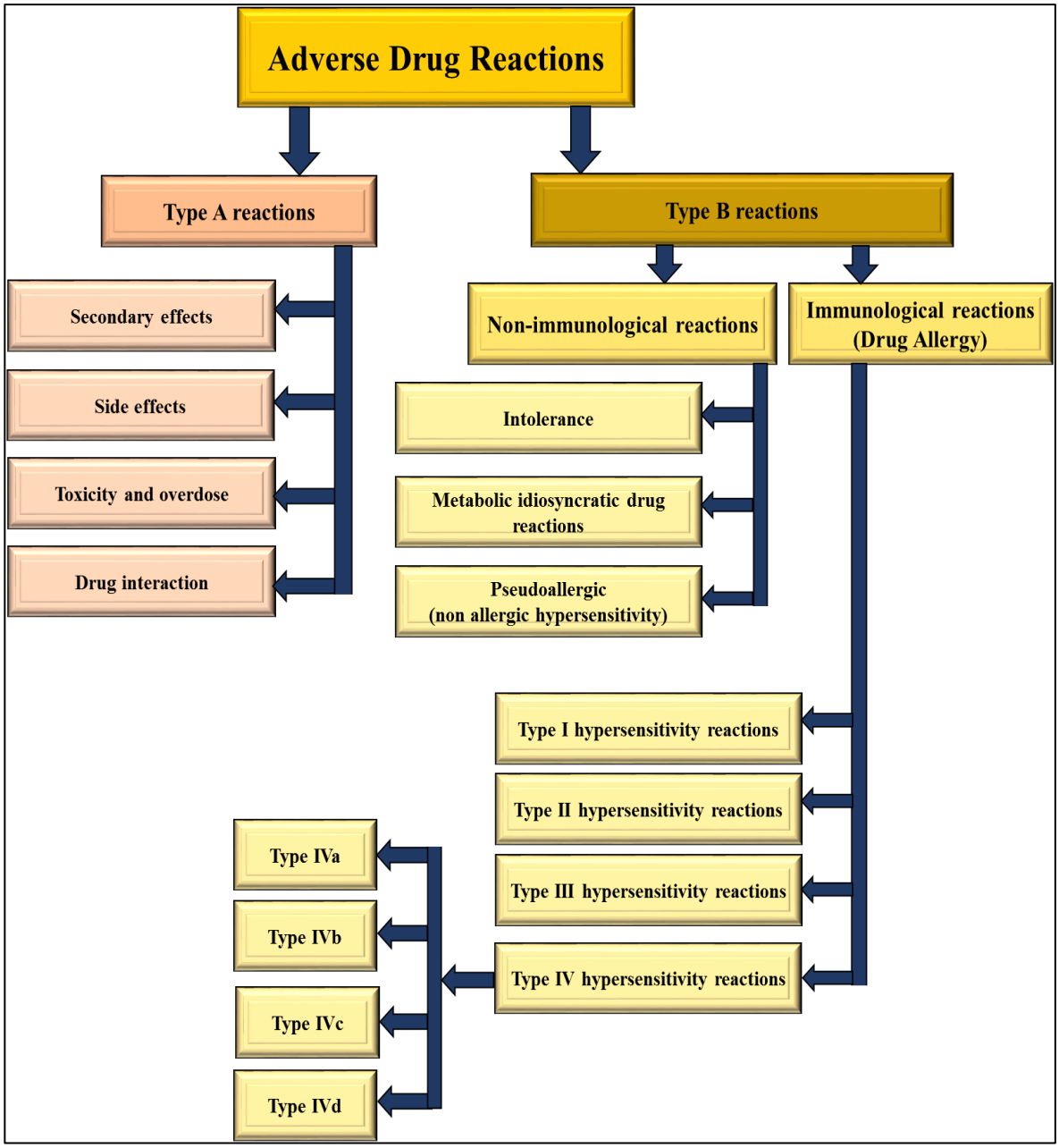


Figure 1-11Classification of adverse drug reactions (ADRs).

1.3.3.1 Type A reactions (on-target).

Type A reactions are considered to be predictable pharmacological reactions (Rawlins MD and JW, 1991). Type A reactions constitute 80% of ADRs (Shah et al., 2005). These reactions are also called on-target, they are expected and common reactions, related to the drug dose, and they depend on the pharmacological actions of the drug. The majority of type A ADRs represent an exaggeration of the desired pharmacological actions of the drug. This

type of reaction mainly occurs due to some error in dosing or sometimes it occurs due to sensitivity of the patient or interactions among drugs in a patient who is treated with different types of drug (Gomes and Demoly, 2005, McDowell et al., 2011).

Type A reactions can be further classified and subdivided according to Rawlins and Thompson classification (Rawlins and Thompson, 1991, Shah et al., 2005).

1.3.3.1.1 Toxicity or overdose.

Overdose is a serious, harmful, and sometimes fatal toxic reaction. Overdose means ingestion of more than a treatment dose of a drug by mistake or intention or due to an exaggeration of the drug's primary pharmacological action (Bennett et al., 2012).

1.3.3.1.2 Side effects.

Side effects means that drug-specific effects or symptoms appear during the treatment, which are other than intentional actions, whether useful, neutral or hurtful (Ferner and Butt, 2008). Side effects could in the most extreme cases lead to death (Lazarou et al., 1998), or they could be one of the reasons for failure in treatment (Hay et al., 2014). The coumarins and heparins are used as anticoagulants for temporary or long-term use. These drugs may have very strong and devastating side-effects, including widespread skin necrosis. There is a case report of an elderly patient who developed severe skin lesions due to the use of heparin for a surgical procedure and after further investigation, they detected that the blood was forming circulating antibodies directed against heparin platelet factor 4 (Sanchez-Politta et al., 2006). There has been a report of another case of sedation with antihistamine treatment (Ferner and Butt, 2008).

1.3.3.1.3 Secondary effects.

Secondary effects are effects that yield from the use of a drug after a short period of treatment as an indirect result of the primary action. For example, diarrhoea cases after treatment with

antibiotics because these drugs will change the gastrointestinal bacterial flora (Shah et al., 2005). Acute watery diarrhoea and abdominal pain may result from treatment with clarithromycin (Namiki and Kobayashi, 2018).

1.3.3.1.4 Drug Interactions.

Drug-drug interactions are wanted or unwanted chemical, pharmacokinetic, or pharmacodynamic reactions, when two or more drugs or chemical substances are used for treatment and come together in the body (Chan et al., 2016). The interaction of drugs are very complex (Pai and Bertino, 2015). For example, that the second drug could influence the metabolism of the first drug or affect the drug's concentration in the bloodstream affecting it's primary interaction with the pharmacological target (Brody, 2018).

1.3.3.2 Type B reactions. (Idiosyncratic / off-target).

This type of reaction is unpredictable, infrequent, and more or less doesn't depend on the therapeutic dose and is not connected to the pharmacological effects of the drug. Type B reactions account for 10-15% of ADRs and the reaction mechanisms are more complex and less well understood than type A reactions. Most of these reactions are related to the activation of the host's immune system by the drug and genetic effects influence susceptibility (Gomes and Demoly, 2005, Shah et al., 2005, Rawlins and Thompson, 1991). Type B reactions have a very complex burden on pharmaceutical manufacturers (Park et al., 2011b). Type B reactions are also subdivided according to Rawlins and Thompson classification (Rawlins and Thompson, 1991), as immunological and non-immunological reactions.

1.3.3.2.1 Non-Immunological Reactions.

These reactions are further divided as following:

A. Intolerance: Mainly occurs due to a change in threshold for drug pharmacodynamics in some patients. When the threshold becomes very low, drugs will produce intolerance (Bennett et al., 2012).

B. Pseudoallergic (Nonallergic Hypersensitivity): Pseudoallergy is a typical non-immune anaphylactic reaction. The responses are not dependent on antigen-specific immune responses. Pseudoallergy often emerges with the first dose of a medication, therefore this reaction is considered unpredictable and occasionally has a lethal consequence (Zhang et al., 2018). Drugs directly react with inflammatory cells such as mast cells, eosinophils and basophils (Pichler et al., 2015). These reactions are similar to anaphylaxis symptoms but the mediator release mechanism from basophils and mast cells does not involve IgE antibodies (Zhang et al., 2018, Pichler, 2007). Some drugs have the ability to induce mast cell degranulation and alter enzymatic activity via an interaction with the MRGPRX2 receptor on mast cells, leading to IgE-independent mast cell degranulation (McNeil et al., 2015, Zhang et al., 2018). There are several drugs that are commonly associated with pseudoallergenic reactions, for instance, opioid drugs and nonsteroidal anti-inflammatory drugs (Zhang et al., 2018).

C. Metabolic idiosyncratic drug reactions (mIDRs): Metabolic idiosyncratic drug reactions come from toxic metabolites followed by covalent binding to cellular proteins (Zuniga et al., 2012). These abnormal reactions to a drug do not occur in all patients. The adverse effects are not related to the pharmacological actions of the drug. They are usually mediated by reactive drug metabolites after the bioactivation in the liver by cytochrome P450 enzymes (Larrey, 2002, Huang et al., 2002). The drug metabolites can be electrophilic chemicals or free radicals which promote a variety of chemical reactions, including depletion of reduced glutathione; covalent binding to proteins, lipids, or nucleic acids; or lipid peroxidation. All of these reaction's produced by the metabolites have direct effects affects

on the biochemistry of the cells on organelles, such as mitochondria, the endoplasmic reticulum, the cytoskeleton, microtubules, or the nucleus (Kaplowitz, 2002). Drug effects could also be indirect on cellular organelles, which leads to activation and inhibition of signalling kinases, transcription factors, and gene-expression profiles. Both direct and indirect intracellular effects leads to cell stress and then cell death (apoptosis) or swelling and lysis (Kaplowitz, 2004, Kaplowitz, 2002). A well-known example of these reactions is acetaminophen (N-acetyl-p-aminophenol, paracetamol (APAP). APAP is the most common drug used as pain killer to remove and treat fever (Lee, 2017). APAP metabolism occurs within liver by three pathways. 90% of APAP is metabolized by phase II metabolic pathways through conjugation. 10% of APAP is funnelled by hepatic cytochrome CYP 2E1 to phase I oxidation, which produces a highly reactive and toxic metabolite, N-acetyl-para-benzo-quinone imine (NAPQI). While phase III pathways include metabolite transport in the form of biliary excretion (Yoon et al., 2016).

1.3.3.2.2 Immunological reactions (Drug Allergy).

Drug allergy was orginally classified into four types by Gell and Coombs in 1963 (Table 1.2) (Rajan, 2003, Baldo and Pham, 2013).

- ***Type I hypersensitivity reactions.*** (IgE-mediated immediate hypersensitivity). These reactions are also called anaphylactic hypersensitivity. The reaction appears after 30-60 min, but it may take place much more quickly after drug exposure. IgE antibodies are the mediators of immediate hypersensitivity reactions. Hypersensitivity develops when the allergen binds to the IgE on the surface of mast cells and basophils. This will lead to degranulation and the secretion of histamine, kinins, and prostaglandins. These substances act together to produce symptoms of immediate hypersensitivity such as increased vascular permeability, vasodilatation, smooth muscle contraction and oedema (Baldo and Pham, 2013, Grant et al., 2007). Chemical and lipid

mediators produce these immediate symptoms while cytokines will cause delayed symptoms (Rajan, 2003). These symptoms appear with cases of urticaria, anaphylactic shock and asthma (Bennett et al., 2012).

- ***Type II hypersensitivity reactions.*** These reactions are known as antibody-dependent cytotoxicity (Baldo and Pham, 2013), or antigen-antibody interactions (Rajan, 2003). IgG or IgM antibodies are responsible for targeting membrane-associated antigens (Male et al., 2012, Beenhouwer, 2018). A sensitization phase leads to production of antibodies. The effector phase, occurs when the antibodies coat the target cells (Beenhouwer, 2018). This causes cell demolition and destruction by complement system activation or due to the cytotoxic effect of natural killer cells especially those with Fc-IgG receptors or by cells such as macrophages. Target cell lysis occurs by lysosomal enzymes and the cytolytic molecule perforin (Pichler et al., 2010).
- ***Type III hypersensitivity reactions.*** These reactions are also known as immune complex hypersensitivity. These reactions occur due to soluble immune complexes which are formed by antigen-antibody interactions. IgG antibodies are mostly responsible for this type of hypersensitivity; however, sometimes IgM antibodies are involved. These antigen-antibody complexes will precipitate in tissues, producing tissue damage. This process initiates complement activation, which will initiate inflammatory and allergic processes such as mast cell degranulation, leukocyte chemotaxis, and lysosomal enzyme release (Baldo and Pham, 2013). The classical type III hypersensitivity reaction is systemic lupus erythematosus (Male et al., 2012).
- ***Type IV hypersensitivity reactions.*** Delayed-type or cell-mediated hypersensitivity. This type of reaction differs from the previous three types because T-cells bring about the adverse reaction (Beenhouwer, 2018). The reactions are so called because the development of clinical symptoms can take one or more weeks to develop (Male et

al., 2012). T-helper and T-cytotoxic cells are responsible for the generation of these reactions (Aronson, 2012, Adam et al., 2011). An interaction between T-cells, antigen presenting cells and a drug causes T-cell activation, proliferation and cytokine secretion (Actor, 2014).

Type IV hypersensitivity reactions are divided into several subtypes based on cytokines secreted and the types of cells involved (Table 1.2) (Adam et al., 2011, Posadas and Pichler, 2007).

The subtypes of type IV hypersensitivity reactions are:

- **Type IVa**

When $CD4^+$ Th1 cells encounter specific antigens, they will be activated and then cause macrophage stimulation by IFN-γ secretion. This leads to a sequence of immune events that sometimes involves $CD8^+$ T-cell responses.

- **Type IVb**

In these reactions, $CD4^+$ Th2 cells produce IL-4, IL-13 and IL-5 cytokines after exposure to drug antigens. Activated B-cells enhance secretion of IgE and IgG antibodies. This will reduce macrophage activity and promote mast cell and eosinophil responses.

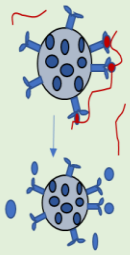
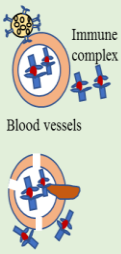
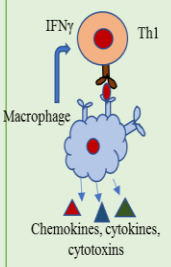
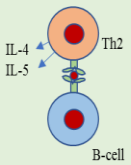
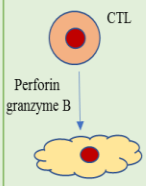
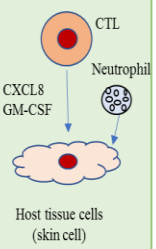
- **Type IVc**

In this type of reaction, $CD8^+$ T-cells act as effector cells. They move to the tissues and kill the host tissue cells. For example hepatocytes or keratinocytes are killed by $CD8^+$ T-cells through secretion of several types of cytolitic molecules including perforin, granzyme B and/or FasL.

- **Type IVd**

In this type of reaction, $CD8^+$ cytotoxic T-cells are again the primary mediators. CXCL8 and GM-CSF producing T-cells will activate neutrophils and produce neutrophilic infiltrates, leading to pustular exanthema. This is a typical case of inflammation of the skin, in acute-generalized exanthematous pustulosis (Adam et al., 2011, Warrington, 2012). CXCL8 chemokins recruit neutrophils (Lyons et al., 2004) and GM-CSF prevents neutrophil apoptosis (Britschgi et al., 2001).

Table 1-2 Gell and Coomb's classification system adapted from (Pichler, 2007).

	Type I	Type II	Type III	Type IVa	Type IVb	Type IVc	Type IVd
Immune reactant	IgE	IgG	IgG	IFNγ ,TNF-α, (Th1 cells)	IL-5, IL-4, IL-13 (Th2 cells)	Perforin, granzyme B (CTL)	CXCL8, GM-CSF (T-cells)
Antigen	Soluble antigen	Cell or matrix associated antigen	Soluble antigen	Antigen presented by cells or direct T-cell stimulation	Antigen presented by cells or direct T-cell stimulation	Cell associated antigen or direct T-cell stimulation	Soluble antigen presented by cells or direct T-cell stimulation
Effector	Mast cell activation	FCR+ cells (phagocyte, NK cells)	FCR + cells Complement	Macrophage activation	Eosinophils	T-cells	Neutrophils
							
Example of hypersensitivity reactions	Allergic rhinitis, asthms, systemic anaphylaxis	Hemolytic anemia, thrombocytopenia	Serum sickness, arthus reaction	Tuberculin reaction, contact dermatitis	Chronic asthma, Chronic allergic rhinitis	Maculopapular, exanthema	AGEP, Behcet disease

1.4. Mechanisms of T-cell mediated drug hypersensitivity.

Several hypotheses have been proposed to describe the development of drug allergy or drug hypersensitivity. These include hapten / prohapten and pharmacological interactions of drugs

with immune receptors (P-I) mechanisms (Pavlos et al., 2015). These are described in detail below.

1.4.1 The hapten/pro-hapten hypothesis.

Certain drugs and/or their reactive metabolites have the ability to bind covalently to proteins. The hapten is a small molecule that interacts with a protein carrier to eventually form a stable hapten-peptide-complex on the MHC of the APCs (Budinger and Hertl, 2000, Naisbitt et al., 2000, Schnyder and Pichler, 2009).

Landsteiner and Jacobs were the first to develop this hypothesis in 1935, when they noticed that it was not possible to activate an immune response by small molecules unless the molecules were chemically reactive and bound to a protein. They coined the term, "hapten". This name comes from the Greek **"hapten"**, meaning "to fasten". Haptens are low molecular weight (LMW) chemicals, they weigh less than (1000 daltons). This reactive molecule needs to bind to another molecule called a carrier. This binding makes hapten molecules antigenic (i.e. to have the ability to produce an immune response) (Figure 1.12 A) (Landsteiner and Jacobs, 1935). The β-lactam antibiotics are the best example for the haptenation phenomenon (Levine, 1960, Ariza et al., 2015, Whitaker et al., 2011). Formation of hapten-protein complexes with β-lactam antibiotics occurs following when the nucleophilic opening of the β-lactam ring. The haptenic structure then binds to protein and forms a drug-protein conjugate (Faulkner et al., 2014). Covalent bonds have energies ranging from 200 to 420 kJ/mol compared to hydrophobic, dipolar, and ionic interactions with bond energies <50 kJ/mol. This type of binding energy enables covalent adducts to survive the intracellular antigen processing of the haptenated protein into short peptides for cell surface expression by MHC complexes (Chipinda et al., 2011). There are other types of binding between the hapten and carrier, such as disulphide exchange and coordinate covalent binding (Chipinda et al., 2011). After binding of a hapten to a carrier protein, the complexes are processed

inside APCs and presented as a stable hapten-peptide complex by the MHC on APCs in the lymph nodes and on APCs settled in the tissues, (See section 1.2.5). These complexes are recognised by naïve T-cells, stimulating clonal expansion (Naisbitt et al., 2000). After the first exposure, primed T-cells develop functionally to two subclasses; the effector T-cells (Teff) and the effector memory T-cells (Tem). These specific T-cells (Teff and Tem) will move to the place of hapten-carrier compound exposure at the time of primary sensitisation (Schnyder and Pichler, 2009, Ebert et al., 2005).

Some drugs have characteristics that differ from haptens. Therefore, they induce sensitisation via a different pathway. These drugs do not bind to protein themselves but they need to be metabolised to a more active substance (via a bioactivation process). These drugs are called **pro-haptens**. After metabolism, they will have the ability to bind to proteins leading to formation of a prohapten-carrier complex, to induce an immune response (Figure 1.12 B). Unlike penicillin, sulfonamides must undergo metabolic transformation into chemically reactive metabolites before a drug-induced response is triggered. Sulfamethoxazole is the archetypal model of a prohapten. This drug does not bind to proteins directly but it is metabolised in the liver by the cytochrome CYP2C9 isoenzyme to a pro-reactive hydroxylamine metabolite (Cribb and Spielberg, 1992). This hydroxylamine metabolite is a more active substance than the parent drug and it will undergo spontaneous oxidation to produce nitroso-sulfamethoxazole (Naisbitt et al., 1999). The nitroso compound binds irreversibly to serum and cellular proteins (Naisbitt et al., 2002, Callan et al., 2009, Sanderson et al., 2007). Reactive drugs and metabolites also induce cell stress and this lead scientists to consider Matzingers Danger model in the context drug hypersensitivity (Williams et al., 2002, Pirmohamed et al., 2002). According to this model, the immune system does not care about self and non-self, but it just tries to protect the body against a dangerous environment (Matzinger, 1994, Matzinger, 2002). Pursuant to this hypothesis, for

any immune response to occur by hapten or prohapten-protein complex, it must be reinforced by another signal called a danger signal. In the absence of a danger signal the hapten complex will be ignored by the immune system (Curtsinger et al., 1999, Matzinger, 1994). Thus, it became clear that the adaptive immune response does not work in isolation from the innate immune system. The innate immune system seems to play a critical role in determining the type of immune response induced by T-cells and B-cells by sending various signals (Gruchalla, 2001). During the sensitization phase of cutaneous hypersensitivity the hapten will interact with keratinocytes, and dermal DCs. The hapten will produce a stress effect on keratinocytes, which leads to release of a group of cytokines and inflammatory molecules such as IL-1β, IL-18, TNFα, and GM-CSF. These molecules work as danger transmitter signals and they support the immune response. As discussed earlier, innate immunity is trigger through pattern recognition receptors such as TLRs. DC activation will produce migration the DCs to the draining lymph node where they mature and present hapten-antigen complexes to naïve T-cells (Erkes and Selvan, 2014, Honda et al., 2013). The first signal is the binding of TCRs on T-cells to MHC molecules on DCs and the second signal is called co-stimulation. This involves the triggering of CD28 on the T-cell by CD80 and CD86 molecules on the DC (or APCs) (See section 1.2.6.4) (Chen and Flies, 2013). Injured cells (stress, necrosis) or cells under infection stress, release danger/alarm signals which increases APCs stimulation, resulting in increased expression of costimulatory molecules. Polarizing cytokines are direct T-cell differentiation factors that are often referred to as signal 3 (Figure 1.12) (Corthay, 2006, Naisbitt et al., 2000, Li and Uetrecht, 2010).

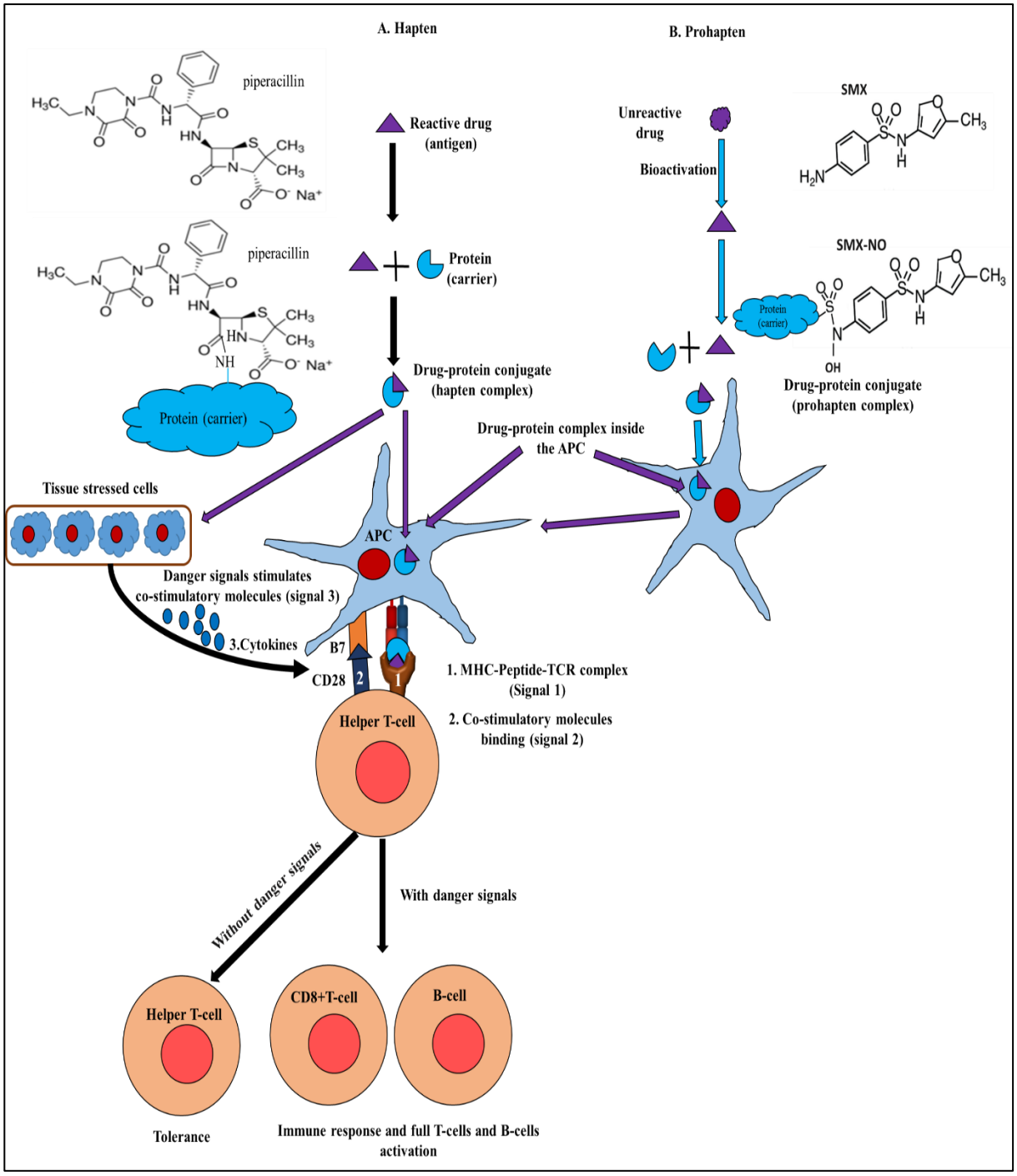


Figure 1-12 Hapten and prohapten hypothesis and the role of danger signals.

1.4.2 The P-I hypothesis (Pharmacological Interaction with Immune Receptors).

This idea came about because some drugs do not have hapten characteristics, therefore they bind directly and reversibly to proteins to stimulate the immune system. This concept is

referred to as the pharmacological interaction of drug with the immune system and gave a mechanistic explanation for many cases of drug hypersensitivity reaction. Moreover, it is based on comprehensive investigations of how T-cell clones from human patients interact with drugs. Pichler (2008) explains that when drugs bind to TCRs on the surface of T-cells this interaction will initiate a pharmacological stimulation. This theory gives an explanation to the appearance of symptoms very quickly in certain forms of hypersensitivities, without previous exposure to the same antigen. Sometimes the response is anarchist although the immune response is very organised (Pichler, 2008). This hypothesis to describe the interaction between small molecules and immune receptors is a new immunological concept and had been defined as the **"direct pharmacological interaction of drugs with immune receptors, the interacting substance is a chemically inert drug that does not have the ability to bind covalently to peptides or proteins, but it can stimulate T-cells if it has affinity for T-cell receptors or MHC-molecules. This binding is reversible** (Pichler, 2013, Pichler et al., 2006, Pichler, 2002). Reversible binding interactions include Van der Waals interactions, electrostatic forces and/or hydrogen bonds. The binding will occur with HLA or the TCR. Therefore, these drugs are less toxic in comparison to hapten drugs (Pichler, 2013, Adam et al., 2011).

According to the P-I hypothesis, there are 3 criteria for T-cells to generate an immune response with a drug; the first criteria is that the T-cells must express a specific TCR that can bind to the drug and induce a stimulatory signal. The second criteria is, the T-cells should have a low threshold for stimulation. The third criteria is that an additional binding of the TCR with the MHC on the APC should exist to increase the intensity of the immune response (Schnyder and Pichler, 2009, Ghosh et al., 2011).

- **There are two shapes of P-I interaction** (Pichler, 2008).

1.4.2.1 P-I TCR (Direct).

In this type of reaction, the drug binds directly to the specific TCR molecule via non-covalent unstable bonds. This will lead the MHC molecule (of APC) to bind to the TCR and induce full activation (Figure 1.13) (Depta et al., 2004, Schmid et al., 2006). Activation of the T-cell can be measured by many methods including, the immediate $Ca2^{+}$ influx into specific T-cells, cytokine synthesis and proliferation (Pichler, 2013). The first examples of this type of interaction were sulfamethoxazole (SMX) and lidocaine. The T-cell clones were activated even if the peptide or the HLA-molecule was removed or replaced (Burkhart et al., 2002, Zanni et al., 1998b). Furthermore, clones responded to the drug in the presence of allogenic MHC alleles, which is referred to as the loss of HLA restriction (von Greyerz et al., 2001b). Moreover, structurally related sulfonamides may block the stimulation of T-cells with SMX. Docking studies have discovered that these blocking sulfonamides bind to the same location as the SMX (CDR3), but without a stimulation signal (Pichler, 2013). Interestingly, only SMX binds to CDR3 with its NH2 group pointing to the peptide-binding groove (Pichler et al., 2015). While other non-stimulatory sulfonamides point their NH2 group toward the TCR and they bind to the outside of peptide interacting site (Werner and Stephen, 2014).

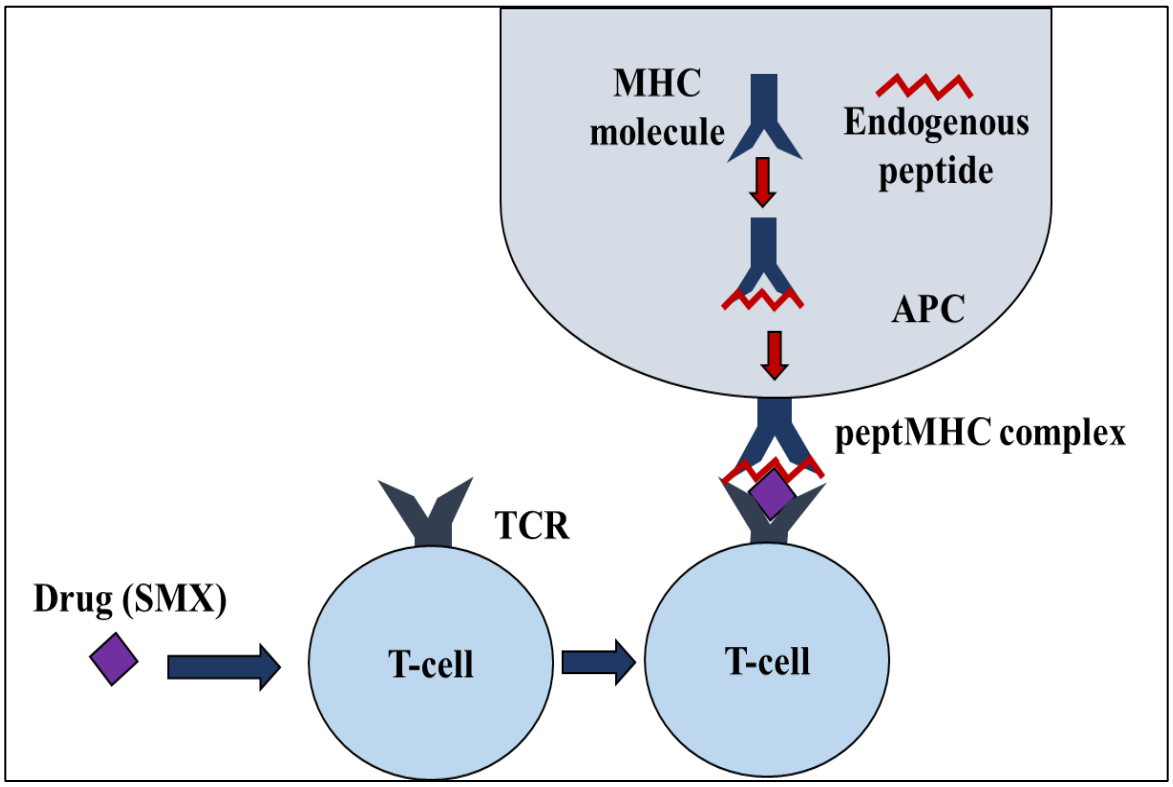


Figure 1-13 P-I TCR (Direct). The drug binds directly to TCR on the surface of T-cell and then that complex binds to MHC molecule on the APC, to generate a comprehensive immune response.

1.4.2.2 P-I HLA (Indirect).

Chung and his group studied Stevens-Johnson Syndrome (SJS) induced by carbamazepine (CBZ), a drug prescribed for the treatment of seizures. SJS is a life-threatening reaction of the skin. Their study found a strong association between a genetic marker, the human leukocyte antigen HLA-B*15:02, and the SJS cases induced by CBZ (Chung et al., 2004a, Leckband et al., 2013). Furthermore, the drug was found to bind directly to the HLA molecule to activate the $CD8^+$ T-cells involved in CBZ-induce SJS. Due to the strength of the HLA-B∗15:02 association with CBZ-induced SJS/association, the National Health Insurance in Taiwan has covered the cost of the genetic screening for HLA-B∗15:02 since 2010, in people treated with CBZ. This test has contributed to the reduction of CBZ-induced

SJS /TEN to almost zero. The test has been used clinically in 23 hospitals in Taiwan (Chen et al., 2011).

There are two models of P-I HLA binding

- **The Altered Peptide Model of P-I HLA (intracellular pathway).**

This model of binding between drugs and HLA was generated from a breakthrough in understanding of the type of connection between the crystallographic structure of the HLA-B *57:01 molecule and abacavir (Ostrov et al., 2012, Norcross et al., 2012). When drugs such as abacavir enter a cell, and are transported to the cytoplasmic reticulum, they bind with endogenous HLA molecules. The drug attaches to a site below the peptide presented by the specific allele HLA-B*57:01, far from the loci peptide-TCR interaction (Figure 1.14 A) (Illing et al., 2012, Ostrov et al., 2012).

Abacavir modifies the structure of HLA-B*57:01 and changes the nature of peptide-binding to HLA-B*57:01. Approximately 20% of the peptides presented by abacavir-modified HLA-B*57:01 are made up novel peptide sequences (Illing et al., 2012). This alteration in the binding capacity of peptides by the new abacavir-modified HLA-B*57:01 will result in the activation of T-cells and hypersensitivity reactions in patients (Illing et al., 2013).

- **The Allo-Immune Model of P-I HLA (extracellular pathway).**

Comprehensive analysis of abacavir-specific T-cells has challenged the altered peptide concept. Further studies have resulted in a new concept, i.e. that the exchange of the peptide is not necessary for immunogenicity, but changing the HLA-(self) allele without alteration the sequence of the peptide is sufficient for immunogenicity (Pichler, 2013). For example abacavir binds directly to the F9-pocket of the peptide-HLA-B*57:01 complexes which is presented on the cell surface. Drug binding occurs directly as a result of the flexibility of the peptide-HLA binding, which allows abacavir to enter the specific binding site below the

peptide, to create (abacavir-peptide-HLA-B*57: 01) complexes without the need of peptide exchange (Figure 1.14 B) (Pichler et al., 2015, Yun et al., 2014).

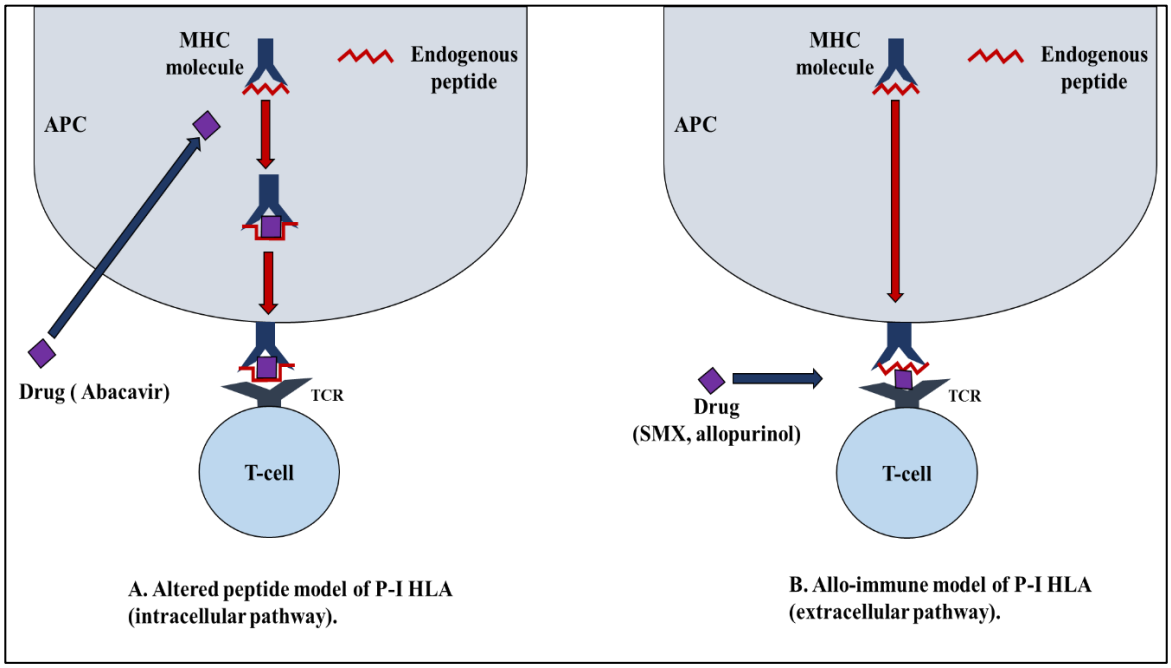


Figure 1-14 P-I HLA concept highlighting 2 possible senarios (A): Altered Peptide Model of P-I HLA (intracellular pathway) the drug enters into the cell and binds to an endogenous HLA, producing an alteration in the peptides bound to HLA molecule. (B): The Allo-Immune Model of P-I HLA (extracellular pathway) the drug such as abacavir binds to the peptide loaded on the HLA molecule. That binding would trigger an altered peptide-HLA complex configuration without a peptide change. In both senarios, the binding will make a change that would be sensed by the TCR as a new antigen that would incite an immune response.

1.4.2.3 The need for co-stimulation signals (danger signals) in P-I reactions.

The cofactors implicated in P-I TCR and P-I HLA may differ. Co-stimulation signals are not an absolute requirement in the P-I HLA activation model especially for abacavir. This is because abacavir-B*57:01 peptide complexes may act as an allo-allele and create a strong direct T-cell stimulation (Pichler et al., 2015). The innate immune system is not stimulated and B-cells are not involved. Therefore, there is no B-cell activation or IgE-mediated reaction confirmed for the P-I mechanism. So far, the drug interaction is limited to the TCR and/or HLA (Pichler, 2003, Yun et al., 2016). On the other hand, the, P-I TCR stimulation

models are different and some may require co-stimulatory factors. For example, activation of T-cells may occur with a concomitant infection. This will lead to upregulation of adhesion molecules and secretion of cytokines, which perhaps lower the threshold of T-cell reactivity needed for hypersensitivity (Pichler et al., 2015).

1.5 The role of HLA human leukocyte antigen in drug hypersensitivity drugs.

Over the last decade many studies explored the specific relationship between drug hypersensitivity and HLA alleles. These studies have enriched and driven the knowledge toward understanding the immunopathogenesis (Pavlos et al., 2012). A strong genetic association between HLA allele and hypersensitivity reactions to certain drugs have been described (Chung et al., 2007). Table 1.3 summarizes some of the well-known examples for drug hypersensitivity cases and the culprit HLA alleles (Fan et al., 2017).

Table 1-3 Drug-induced ADRs and HLA allele associations, adopted from (Fan et al., 2017).

Drug	HLA allele	Phenotype	Population
Carbamazepine	B∗15:02	SJS/TEN	Taiwanese, Han Chinese, Thai, Malaysian, Asian
Carbamazepine	A∗31:01	SJS/TEN	Han Chinese Caucasian Japanese
Carbamazepine	B∗15:11	SJS/TEN	Japanese
Allopurinol	B∗58:01	SJS/TEN	Han Chinese Caucasian, Thai Japanese
Allopurinol		SCARS	Taiwanese
Phenytoin	B∗15:02	SJS/TEN	Han Chinese, Thai
Lamotrigine	B∗15:02	SJS/TEN	Han Chinese
Nevirapine	DRB1∗01:01	DRESS	Hispanics, African
Sulphamethoxazole	B∗38	SJS/TEN	European
Amoxicillin-clavulanate	DRB1∗15:01-DQB1∗06:02	DILI	Caucasian
Flucloxacillin	B∗57:01	DILI	Caucasian
Lumiracoxib	DRB1∗15:01	DILI	Not available
Ticlopidine	A∗33:03	DILI	Japanese

1.5.1 Human Leukocyte Antigen (HLA).

The specific genetic loci implicated in immune responses is known as the major histocompatibility complex (MHC). This genetic locus MHC encodes for highly polymorphic cell surface molecules. In humans, the HLA (Human Leukocyte Antigen) system is a gene complex encoding the MHC. This genetic complex HLA is located on the short arm of chromosome 6. Human MHC is divided in three parts (Figure 1.5) (Choo, 2007, Boegel et al., 2018). As we explained before (in section 1.2.5.1, figure1-5) MHC I molecules are encoded by MHC-A, B and C, and these are expressed on all nucleated cells types but to different degrees. MHC-II molecules are encoded by MHC-DP, DQ and DR, which are expressed on APCs and on some endothelial tissue, thymic epithelial cells and B-cells (Degoot et al., 2018, Choo, 2007, Mosaad, 2015). Nomenclature for HLA has been generated by the Nomenclature Committee for Factors of the HLA System of the World Health Organization (WHO). They use separators to divide the group of alleles (Figure 1.15) (Torres and Moraes, 2011).

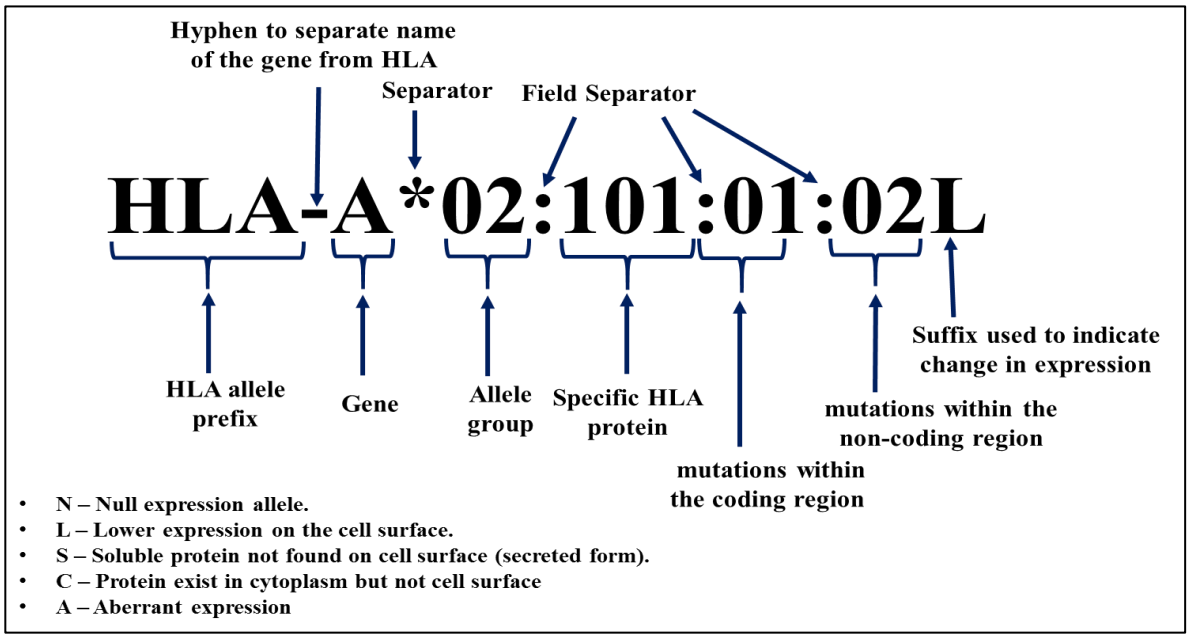


Figure 1-15 Nomenclature system for the HLA system.

1.6 Clinical cases.

Drug hypersensitivity can affect any organ but the skin, the liver and blood are the most important targets. Some drugs have a limited action on one organ, while, others drugs have a wide range of actions and affect several organs (Uetrecht and Naisbitt, 2013).

1.6.1 Skin rashs or skin lesions.

1.6.1.1 Maculopapular rash.

Maculopapular or morbilliform rashes are a very common type of drug reaction, which results in skin lesions (drug-induced skin rash). This type of rash constitute approximately 90% of all clinical cases of drug-reactions (Uetrecht and Naisbitt, 2013). Maculopapular rashes start 1-2 weeks after commencing treatment with a drug. This type of rash is not very dangerous and cytotoxic $CD4^+$ T-cells are the dominant cell type for these reactions (Naisbitt, 2004).

After stimulation of $CD4^+$ T-cells and their infiltration into cutaneous tissue, these cells will mediate skin inflammatory reactions by binding their chemoreceptor CCR10 to the skin-associated chemokine CCL27. This interaction between CCL27 and CCR10 plays a vital role in T-cell recruitment and T-cell- mediated skin inflammation (Lee and Rosenberg, 2013, Homey et al., 2002).

1.6.1.2 Urticaria.

Urticaria is dermal oedema caused by vasodilatation, and perivascular nonnecrotising infiltrates; comprising primarily of mononuclear cells, predominantly $CD4^+$ lymphocytes (Jain, 2014). These reactions are also called hives. Urticarial lesions are itchy (Levine and Price, 1964). Urticaria occurs mainly due to IgE-mediated allergic reactions. Penicillin reactions are examples of this type of adverse event. Sometimes, urticaria may not be a real allergic reaction because some people develop it when they feel cold or following exercise.

Urticaria is clinically characterised by large, raised, pruritic skin lesions that do not continue for more than 24 hrs (Uetrecht and Naisbitt, 2013). Some drugs such as opiates or codeine have the ability to activate mast cells. There are other drugs that produce pseudoallergic reactions that may include urticaria, such as angiotensin converting enzyme (ACE) inhibitors, aspirin and nonsteroidal anti-inflammatory drugs (NSAID). They may produce or exacerbate chronic urticaria by their pharmacological mechanisms which involves arachidonic acid metabolism (Mathelier-Fusade, 2006).

1.6.1.3 Fixed Drug Eruption.

This is a type of skin eruption is characterised by erythematous round lesions with oedematous plaques that are brown to dark purple or red in colour (Pal et al., 2014). The reaction is mediated by $CD8^+$ memory T-cells that are limited to the site of the lesions. The site of the skin eruption is fixed. After re-exposure to the drug, the lesion will appear at the same place (Pal et al., 2014). Positive patch tests results are observed for this condition, if the test is applied on the site of the lesion (Uetrecht and Naisbitt, 2013). Co-trimoxazole is the most common example of this type of reaction (Miah et al., 2008).

1.6.1.4 Drug reaction with eosinophilia and systemic symptoms (DRESS).

DRESS syndrome is a serious life-threatening condition characterized by skin eruption, fever, haematologic abnormalities, and multi-organ involvement (Walsh and Creamer, 2011). The first detection of these cases was with anticonvulsants drugs. Therefore, these reactions were given the name of anticonvulsant hypersensitivity syndrome (Shear and Spielberg, 1988). Later these reactions were given another name, drug reaction with eosinophilia and systemic symptoms (DRESS) which is more commonly used nowadays (Uetrecht and Naisbitt, 2013).

This condition (DRESS) is characterised by a wide spectrum of clinical features, and the extended latency period, which leads to a delay in diagnosis. The main symptoms are fever, rashes, lymphadenopathy, and more severe symptoms resulting from hepatitis, nephritis, pneumonitis, carditis, thyroiditis, and hematologic illnesses such as (eosinophilia, atypical lymphocytes, thrombocytopenia, or leukopenia). However, skin signs and haematological abnormalities usually lead to the diagnosis of the case of hypersensitivity to the culprit drug (Walsh and Creamer, 2011, Uetrecht and Naisbitt, 2013, Um et al., 2010). Patients with DRESS syndrome present variable clinical and pathological features. Reactivation of viruses such as human herpesviruses (HHVs) is also a hallmark of DRESS (Cho et al., 2017). DRESS is possibly associated with proliferation of activated $CD8^+$ T lymphocytes directed against viral antigens (Liang et al., 2018, Drago et al., 2016). Carbamazepine, phenobarbital, and sulfonamides are common examples of DRESS inducing drugs (Seishima et al., 2006).

1.6.1.5 Steven Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN).

These lesions are more severe than any other type of skin reaction. SJS produces about 10% of epidermal detachment, while TEN about 30%. The clinical features start with fever and malaise. This is followed by a painful rash. Blisters are a classic sign which is known as Nikolsky's sign; the tissue lesions histologically will show keratinocyte apoptosis with separation between the dermis and epidermis. The symptoms start in a much shorter time shorter than time DRESS (7-14 days). This could be even quicker if the patient was re-exposed to the causative drug (Uetrecht and Naisbitt, 2013, Cote et al., 1995, Mustafa et al., 2018). The mediators for these reactions are cytotoxic molecules (perforin, granzyme B, and granulysin), which are found in a high density in the lesions (Schlapbach et al., 2011, Posadas et al., 2002). Moreover, the cells in the skin lesions mainly consist of cytotoxic T lymphocytes and NK cells. Stimulation of the Fas ligand (Fas l) pathway will produce diffuse

keratinocyte apoptosis, followed by epithelial necrosis. Other inflammatory molecules like IFN-γ, TNFα, soluble Fas l, and NK-activating molecules, are found within the skin lesions (Mustafa et al., 2018, Ang et al., 2013).

1.6.2 Idiosyncratic drug induced liver injury (iDILI).

Idiosyncratic drug-induced liver injury (DILI) is an infrequent adverse drug reaction. It can produce jaundice, liver failure, or even death (Chalasani et al., 2014). iDILI is an important disease that occurs following drug use and it is very challenging for both clinicians and drug developers because the appearance of this reaction is determined by characteristics of the individual patients, and its incidence is very low (e.g., 1 in 10,000). However, its consequences are very severe. Patients may develop liver failure with the risk of death (Benesic et al., 2018). Age is one of the important factors influencing susceptibility. Patients with chronic hepatitis (Types A, B, or C), are more likely to develop iDILI especially in patients treated with isoniazid and antiretroviral drugs (Yamashita et al., 2017). In a genetic study of iDILI causes, several HLA were found to be associated with iDILI. Examples include flucloxacillin, co-amoxiclav, ximelagatran, lapatinib, lumiracoxib. They found that several HLA haplotypes, B*57:01, DRB1*15:01, DRB1*07:01, DQA1*02:01 and DRB1*15:01 were risk factors in diverse ethnic of patient groups (Alfirevic et al., 2012). More recently, drug-specific T-cells have been detected in patients with flucloxacillin and co-amoxiclav-induced-iDILI (Cho and Uetrecht, 2017, Tailor et al., 2015).

1.6.3 Hematologic Idiosyncratic Adverse Reactions.

1.6.3.1 Drug-induced immune hemolytic anaemia (DIHA).

It is not a common condition but still a dangerous type of ADRs. The mechanisms implicated in this condition are not fully understood. The reaction is thought to occur following oxidative damage to vulnerable erythrocytes. This happens when oxidative-stress-

susceptible red blood cells face drug-induced oxidative damage, through the drug or its metabolites. Many drugs produce this reaction, especially antibiotics, but the reasons are considered to be very confounding (Bubp et al., 2015, Renard and Rosselet, 2017).

1.6.3.2 Drug-induced thrombocytopenia.

All idiosyncratic drug-induced thrombocytopenia reactions are thought to be immune mediated, but the mechanisms may differ (Uetrecht and Naisbitt, 2013). This condition is characterised by a decrease in the platelet count to below 150000 platelets/μL. If the platelet count drops below 10000 platelets/μL, the patient will be at risk of life-threatening haemorrhage (Aster, 2010). Heparin is a very well-known example of this condition. Antibodies against the heparin-platelet factor 4 complex have been detected in patients suffering from thrombocytopenia (Warkentin, 2003).

1.6.3.3 Drug-induced agranulocytosis.

Agranulocytosis is one of the lethal idiosyncratic reactions that occurs when neutrophil counts drop to less than 500 neutrophils/μL in the blood. This will produce dangerous and fatal symptoms. There is a large group of drugs that produce this condition, such as analgesics, antipsychotics, antithyroids and anticonvulsants (Andres et al., 2006). Clozapine is an antipsychotic drug used in treatment of refractory schizophrenia. This drug produces neutropenia and agranulocytosis in 0.8-3% of patients. Clozapine undergoes bioactivation to reactive metabolites by the cytochrome P450 and peroxidase enzymes (Pirmohamed et al., 1995). This will cause neutrophil cytotoxicity. The mechanism of inducing the agranulocytosis by clozapine is not very clear (Pirmohamed and Park, 1997).

1.7 Diagnostic tests.

Drug hypersensitivity reactions are unpredictable, severe, and life threatening. Therefore, the diagnosis is a critical step to control and prevent it. These reactions are either fast and

start within 1-6 hrs after initiating the treatment with a causative drug (immediate reactions) or the reactions are delayed by several days or more (delayed reactions), for example, exanthematous eruptions (Brockow et al., 2015).

There are two types of diagnostic test, either in the body of a living organism referred to as in vivo and / or in a test tube, which referred to as in vitro. Examples of in vivo diagnostic tests include skin tests and eosinophil count and the presence of autoantibodies. In vitro tests include the analysis and detection of drug-specific T-cells. Furthermore, in vitro tests can measure expression of certain cytokines that are produced from drug-stimulated PBMCs during the activation by drugs (Gómez et al., 2012).

1.8 Treatment of Hepatitis C (HCV).

Hepatitis C virus (HCV) is a serious and potentially life-threatening viral infection that affects an estimated 71 million individuals worldwide. Even though, a quarter of the infected patients effectively clear the virus (WHO, 2017), the majority of patients develop chronic HCV infection. Inflammation in the liver leads to severe liver sicknesses, including hepatocellular carcinoma, liver fibrosis and cirrhosis. Therefore, hepatitis C is the most common indicated condition for liver transplantation in the USA (Verna and Brown, 2006a). Chronic HCV infection is responsible for 300,000 to 400,000 deaths per year, due to cases of cirrhosis, end-stage liver disease and hepatocellular carcinoma (Hoofnagle, 2002).

HCV has traditionally been treated with a regimen of PEGylated IFN-α and ribavirin, which provides a sustained antiviral response in 40-50% of cases. In contrast, an updated triple treatment regimen including telaprevir (TVR, VX-950), increases the frequency of patients that achieve viral control up to 70% (Lang, 2007). TVR is an NS3.4.A protease inhibitor for use against HCV genotype 1 which prevents both the cleavage of viral proteins into active polypeptides and the deactivation of hepatic cellular proteins that are essential for mediating

the interferon cascade and mounting an viral response (Jesudian et al., 2012, Morikawa et al., 2011).

While administered orally as a single S-configurated diastereomer, TVR spontaneously forms the corresponding R-diastereomer which is approximately 30-fold less active pharmacologically (Garg et al., 2012). Despite enhanced viral suppression, the triple regimen is associated with an increased risk of adverse cutaneous reactions, with triple TVR-containing therapy causing severe rashes in 4.8% of patients, compared to just 0.4% of patients treated with the standard dual therapy. Of more concern is that a small subset of patients treated with TVR develop life-threatening cutaneous drug hypersensitivity reactions including DRESS and SJS (Roujeau et al., 2013a). This eventually lead to withdrawal of the drug.

- **Telaprevir (TVR).**

TVR was approved by the FDA in May 2011. The TVR dose is 750 mg which is taken three times per day together with peginterferon alfa and ribavirin (Bronson et al., 2012).

There are many side effects related to TVR treatment, such as nausea/vomiting, tasting change, skin lesions including rashes, blisters and itching, skin and mouth sores and irritation of the eyes. Many studies confirmed that treatment with telaprevir in combination with other drugs produces severe skin lesions such as SJS, DRESS and TEN (Nicole Cutler, 2013). When taken orally, TVR has a high rate of absorption in the small intestine. Its plasma concentration reaches maximum after a single dose in 4-5 hrs. This drug is highly bound to plasma proteins (59-76%), it especially binds to alpha 1-acid glycoprotein and albumin with an inverse concentration-dependent pattern (Vertex., 2013). Metabolism of TVR occurs mainly in the liver through reduction, oxidation and hydrolysis, which will produce various metabolites. Figure 1.16 show the structure of TVR and its alternative diastereomer.

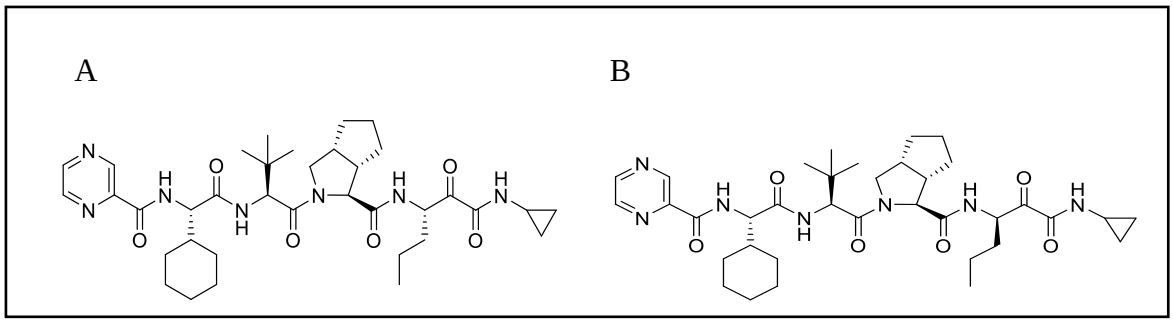


Figure 1-16 (A) Telaprevir (S-diastereomer) and (B) R-diastereomer (PubChem, 2018a).

1.9 Treatment of autosomal dominant polycystic kidney disease (ADPKD).

ADPKD is one of the most common monogenic defects of the kidney, it is widespreaded among different ethnic groups. It occurs in 1:400 to 1:1,000 patients. Tolvaptan as selective arginine vasopressin (AVP) a V2 receptor antagonist, it used in the treatment of ADPKD (Watkins et al., 2015a). The treatment with tolvaptan in ADPKD produces a slow increase in total kidney volume (Torres et al., 2012). The risk of liver failure has been reported in patients receiving long-term tolvaptan therapy, therefore patients who are treated with tolvaptan need continued monitoring through liver function tests (Watkins et al., 2015a).

1.10 Hyponatremia

Hyponatremia is an important and common clinical condition resulting from an electrolyte abnormality. This condition is characterised by a reduced serum sodium level below 135 meq/l when the kidney doesn't have the ability to excrete a water load or excess water intake (Sahay and Sahay, 2014, Eric, 2018). Tolvaptan has been approved by the FDA for management of hyponatremia. This drug is used orally, the dose is usually 15 mg/day. However, the dose may be increased to a maximum of 60 mg daily depending on sodium concentrations (Page, 2011). Tolvaptan is used also to treat hyponatremia because of its

ability to work as a selective arginine vasopressin (AVP) V2 receptor blocker (Dixon and Lien, 2008).

- **Tolvaptan.**

Tolvaptan can be given as single oral doses ranging 60 to 480 mg/day. After its administration orally it will be absorbed by the gastrointestinal tract and its bioavailability is 50% after a 30 mg dose. Tolvaptan is mainly metabolised by cytochrome P450 3A and forms two types of metabolites; dehydrogenated and hydroxylated forms (Shoaf et al., 2012, Lu et al., 2016). The main two metabolites for tolvaptan are DM-4103 and DM-4107 (Figure 1.17), they are excreted in urine and feces, respectively (Lu et al., 2016).

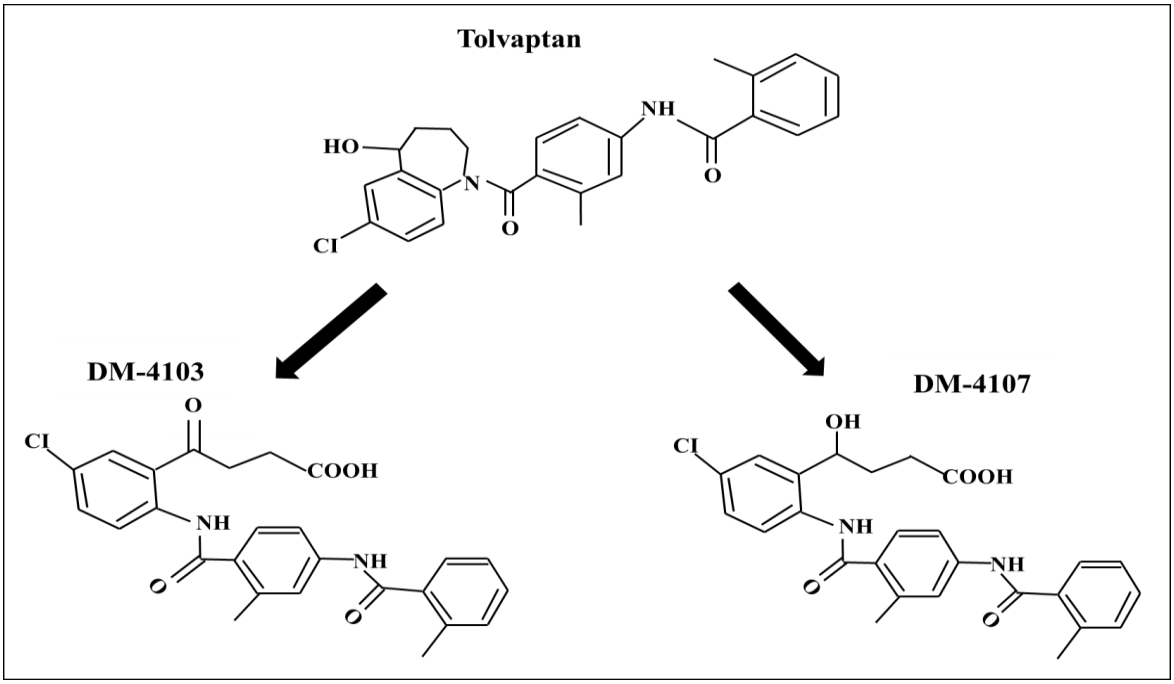


Figure 1-17 Tolvaptan and the main metabolites DM-4103 and DM-4107 (Wan et al., 2012).

Mosedale et al studied the effect of tolvaptan on the liver. They found that the liver could be a target for an adaptive immune attack, which will lead to clinically significant liver injury. They suggested that iDILI starts when the drug induces hepatocyte stress, which will lead to

a release of danger signals and stimulation of the innate immune system. These steps are important for activation but they not enough to activate the adaptive immune response in the liver (Mosedale and Watkins, 2017, Mosedale et al., 2018). Since tolvaptan is metabolised in the liver by the CYP3A4 system (Dixon and Lien, 2008), the concentration will increase with any co-medication that inhibits CYP3A4 activity. Tolvaptan-induced exosomes are released by primary human hepatocytes in patients in the absence of overt necrosis. The secreted exosomes are associated with mitochondrial-induced apoptosis and oxidative stress (Mosedale et al., 2018).

1.11 Treatment of Leprosy.

Leprosy is a disease that is characterised by chronic granulomatous lesions that occur due to infection with Mycobacterium leprae. The bacteria mainly infects the skin and nerves and this lead to immunological damage. The symptoms also include ulcerative lesions of hands, feet and eyes as well as paralysis due to damage of the nerves (Walker et al., 2014). Dapsone is the main drug used in leprosy treatment. However, this drug is also used to treat cases of refractory skin lupus (Lahita, 2007).

- **Dapsone (DDS).**

DDS (4, 4-diaminodiphenylsulfone), is used as oral tablets in doses ranging from 25 mg to 100 mg daily. DDS tablets have a different commercial name in different countries, for example, Dapsone® (Australia), Daps® (Argentina), Avlosulfon® (Canada), Dapson®, (Denmark, Egypt, the Netherlands, Norway), Dapson-Fatol® (Germany). DDS is used to treat leprosy usually with another anti-leprosy drugs (which are rifampicin and clofazimine (Venkatesan, 1997)). DDS it is also used in prophylaxis treatment against toxoplasmosis, in HIV and in treating acne vulgaris and other medical conditions. Moreover, DDS has been confirmed to be effective in different immune conditions and inflammatory diseases such as

recluse spider bites, dermatitis, leukocytoclastic vasculitis and rheumatoid arthritis (Bloom and Ryan, 2013).

The mechanism of action of DDS is still not fully understood, but generally, DDS inhibits the synthesis of dihydrofolic acid by competing with para-aminobenzoate at the active loci of dihydropteroate synthetase. However, DDS may also inhibit neutrophil function through prevention of the respiratory burst and inhibition of adherence to the vascular endothelium. The peak serum level of DDS is reached after 2-8 hrs. DDS is highly bound to proteins (70-90%) and it is distributed widely in the body. This drug has the ability to accumulate in the liver, kidneys, skin and muscles. It is mainly metabolised in the liver by acetylation, the dapsone metabolites are removed through the urine by formation of glucuronide conjugates (Bloom and Ryan, 2013). Figure 1.18 shows DDS and its metabolite nitroso (DDS-NO), which is formed through oxidative metabolism.

Recent studies report that DDS induced hypersensitivity occurs in 0.5-3.6% of patients (Kosseifi et al., 2006). There are ongoing attempts to confirm if the parent drug or its nitroso metabolite are the culprits in hypersensitivity reactions (Vinod et al., 2013, Alzahrani et al., 2017b). DDS hypersensitivity is a condition that is characterised by high fever, skin rash, lymphadenopathy, eosinophilia, hepatitis, acute pneumonitis, and other systemic features (Kosseifi et al., 2006).

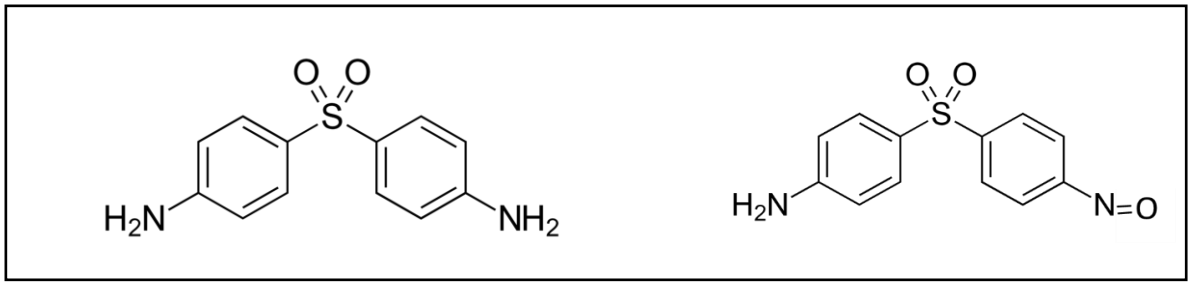


Figure 1-18 A- dapsone structure (Pubchem, 2018b) B- nitroso dapsone (PubChem, 2018c)

1.12 Aims of the thesis.

The primary aim of this thesis is to investigate and discover the immunological aetiology of drug hypersensitivity reactions for 3 different drugs with different pharmacological mechanisms by utilising human blood samples from healthy donors and hypersensitive patients. We generated drug-specific T-cells clones, and utilised a battery of biological and functional assays to explore to chemical and cellular basis of hypersensitivity reaction to telaprevir, tolvaptan and dapsone.

Chapter 2: Materials and Methods.

2.1 Reagents, drugs and chemicals.

- Lymphoprep-source-Axis-Shield (Dundee, UK).
- Magnets, magnetic columns, and antibody-conjugated magnetic bead kits for the isolation of cell subsets from PBMCs including $CD14^{+,}$ pan-T, $CD25^{+}$, or $CD45RO^{+}$ populations-source-Miltenyi Biotec (Surrey, UK).
- A range of CD4 and CD8 conjugated fluorochromes for use in flow cytometry, along with CD1a-fluorescein isothiocyanate (FITC), CD11a-FITC, CD11c- phycoerythrin (PE), CD14-FITC, CD40-FITC, MHC class I-PE, MHC class II-FITC, PD-L1-PE, CD45RO-PerCP-Cy5, and the anti-mouse Ig/ negative control compensation particles set-source- BD Biosciences (Oxford, UK).
- PD-1-PE, CTLA4-allophycocyanin (APC*), TIM-3-PE, CD80-FITC, CD1a-FITC, and CD86-FITC-source-Serotec (Kidlington, UK).
- CD83-PE- source- R&D systems (Minneapolis, USA).
- Galectin-9-APC*-source-Biolegend (Cambridge, UK).
- Materials for blocking studies mouse anti-human HLA-ABC (isotype, IgG1; clone DX17), mouse anti-human HLA-DR/DP/DQ (isotype, IgG2a; clone, Tu39), mouse anti-human HLA-DR (isotype, IgG2a; clone G46-6), and mouse anti-human HLA-DQ (isotype, IgG2a; clone, Tu169) monoclonal blocking antibodies and their respective isotype controls-source-BD Biosciences (Oxford, UK).
- Anti-human HLA-DP blocking antibodies-source-Serotec (Kidlington, UK). LEAF purified (< 0.1 EU/μg endotoxin) mouse anti-human CD274 (PD-L1; isotype, IgG2b; clone, 29E.2A3), LEAF purified mouse anti-human CD273 (PD-L2; isotype, IgG1; clone, MIH18), LEAF purified mouse anti-human CD152 (CTLA4; isotype, IgG1; clone, L3D10), and LEAF purified mouse anti-human CD366 (TIM-3; isotype, IgG1; clone, F38-2E2) were azide-free and purchased from Biolegend (Cambridge, UK).

- ELIspot multiscreen filter plates-source-Millipore (Watford, UK),
- The ELIspot kits containing coating and detection antibodies along with BCIP/NBT Plus liquid substrate solution for IFN-γ, IL-13, IL-17, IL-22, IL-5, granzyme B, and perforin-source-mabtech (Nacka Strand, Sweden).
- The ELIspot kit for Fas ligand-source-Abcam (Cambridge, UK).
- Recombinant human GM-CSF, recombinant human interleukin-2 (rhIL-2), and human IL-4-source-Peprotech (London, UK).
- Foetal Bovine Serum (FBS) and Human AB Serum -source-Invitrogen (Paisley, UK) and Innovative Research (Michigan, USA), respectively.
- The [3H]-methyl thymidine (5 Ci/mmol)-source-Moravek (California, USA)
- Meltilex scintillator sheets, sample bags, and printed glass fibre filter mats for thymidine analysis-source-Perkin-Elmer (Waltham, USA).
- Carboxyfluorescein diacetate succinimidyl ester (CFSE)-source-eBioscience (San Diego, USA).
- Cell culture flasks and nunc 6-, 24-, 48-, and 96-well plates-source-Thermo Scientific (UK).
- Purified tetanus toxoid (TT) -source-Statens seruminstitut (Copenhagen, Denmark). DNA-selective magnetic beads, protease, associated wash buffers and the Magnetic Separation Module-1 for the isolation of DNA-source-Chemagen (Baesweiler, Germany).
- Cyclosporine-A-source-Fluka Analytical (Dorset, UK).
- Dimethyl sulfoxide (DMSO), Roswell Park Memorial Institute (RPMI)-1640, penicillin and streptomycin, transferrin, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer), L-glutamine, Hanks balanced salt solution (HBSS), bovine serum albumin (BSA), ethylenediaminetetraacetic acid

(EDTA), sodium azide, and all other unlisted reagents were purchased from Sigma-Aldrich (Dorset, UK).

- Telaprevir and its metabolites - source- Janssen Pharmaceutica (Belgium).
- Tolvaptan and its metabolites-source-Otsuka Pharmaceuticals (Japan).
- Dapsone and its metabolites -source-Sigma (UK).

2.2 Cell culture medium and Buffers.

- **Cell culture medium -R9 medium** -500 mL RPMI 1640, 100 μg/mL penicillin, 100 U/mL streptomycin, 100 mM L-glutamine, 25 μg/mL transferrin, 10% (v/v) human AB serum, 25 mM HEPES buffer. Used for T-cells clones.
- **Cell culture F1 medium**-500 mL RPMI 1640, 100 μg/mL penicillin, 100 U/mL streptomycin, 10% (v/v) FBS, 25 mM HEPES buffer, 2mM L-glutamine. Used for Epstein-Barr virus (EBVs) culture.
- **MACS buffer (X 10)**-50 mL HBSS, 50 μg/mL BSA, 20 mM EDTA. Used for magnetic bead isolation for cells.
- **FACS buffer** -500 mL HBSS, 10% (v/v) FBS, 0.2 mg/mL sodium azide. Used for T-cells phenotyping.
- **Phosphate buffered saline (PBS; X 10)** - NaCl (80g), Na2HPO4 (11.6 g), KH2PO4 (2g), KCl (2g). Dilute to 1 litre with distilled water, further 1:10 dilution required before use, final pH: 7.0. Mainly used in ELIspot for washing plate.

2.3 Human Subjects.

Up to 120 mL venous blood was collected from healthy volunteers and from clinically diagnosed hypersensitive patients.

The study was approved by the Liverpool local research Ethics Committee and informed written agreements/consents were obtained from all participating blood donors.

2.3.1 Generation of drug-specific T-cells clones from healthy volunteers and from hypersensitive patients by isolation of peripheral blood mononuclear cells (PBMCs).

Blood was taken by venesection and collected in heparinised vacutainer tubes. Blood (25 mL) was carefully layered on lymphoprep (25 mL) and spun at 2000 revolutions per minute (rpm) for 25 min with zero deceleration in a centrifuge at 25°C. Then the blood was separated to different layers that were visible from top to the bottom. They were easily identifiable by their individual colours; plasma (yellow), lymphocyte layer (cloudy white to grey), lymphoprep (transparent), erythrocytes (red). In the middle, the buffy coat layer containing the peripheral blood mononuclear cells (PBMCs) was carefully collected using a Pasteur pipette (Figure 2.1). PBMCs were placed in a new tube, and washed twice with Hanks balanced salt solution (HBSS). The ratio of dilution was 1:4 and then cells were centrifuged at 1800 rpm for 10 min. The supernatant was discarded and the cell pellet resuspended in HBSS. The tubes were then spun at 1500 rpm for 10 min and the cells resuspended in culture medium prior to counting.

PBMCs (10 μL) were mixed with an equal volume of trypan blue (0.2 % w/v) and another (20 μL) of HBSS was added. Then (10 μL) of the mixture was placed on a Neubauer haemacytometer (Sigma-Aldrich) under a Leica DME microscope (Leica Microsystems, Milton Keynes). Dead cells were much darker than the live cells under the microscope. The percentage viability was calculated by the following equation: percentage viability=viable cells ÷ total cells x100. Percentage viability was ≥95% for all the PBMC isolations. Cells were then used in functional assays or frozen for later use by resuspending them in R9 medium (10-20x10^6 cells/mL) and further diluting them 1:1 with “freezing mix” (80% human AB serum, 20% DMSO) in 1.8 mL cryovials (total volume, 1 mL; maximum cell

concentration per vial, $5x10^6$/mL). Cryovials were placed in Mr. Frosty tubs and stored at -80°C for 24-48 hrs before being transferred to -150°C for longer term storage.

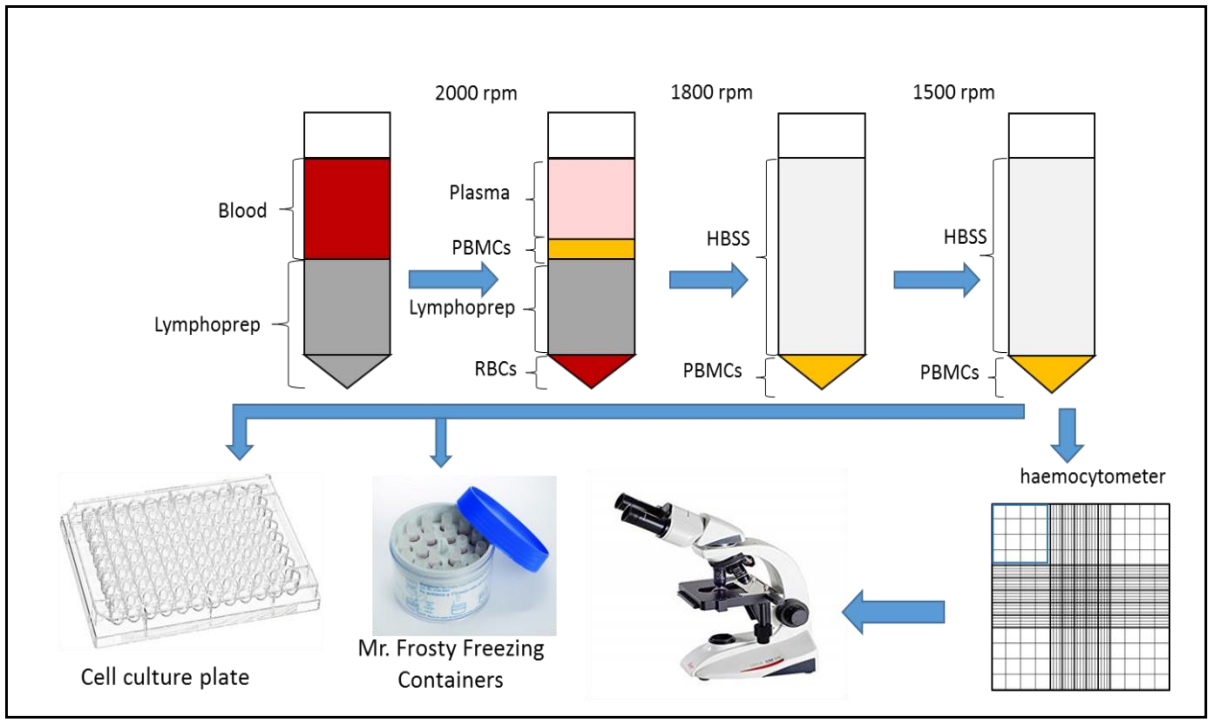


Figure 2-1 The procedure for separation of PBMCs.

2.3.2 Detection of drug-specific PBMC responses using the lymphocyte transformation test (LTT).

PBMCs ($0.15x10^6$ per well) were cultured in triplicate. The following reagents were added:

- Diluted drug concentrations by serial dilution.
- Tetanus toxoid (5 µg/mL) as a positive control.
- Negative control (medium).

The final volume inside the wells was 200 µL for all the conditions. The test plates were incubated for 6 days at 37°C and 5% CO_2. [^{3}H]-thymidine was added for the last 16 hrs (0.5 µCi/well). The cells were then harvested on to paper filter mats which were sealed with wax after drying using tomtec harvestor 96 (Receptor Technologies). Incorporated radioactive

wax was then read using a Microbeta Trilux 1450 LSC (Perkin-Elmer, UK) which interpreted the data as radioactive counts per minute (cpm), to measure the rate of [^{3}H]-thymidine uptake by the cells. This gives a quantitative measure of proliferation in counts per minute (cpm), Figure 2.2 illustrates the procedure with telaprevir. An SI ≥1.5 was considered as an indicator for the proliferation of T-cells with the culprit drug.

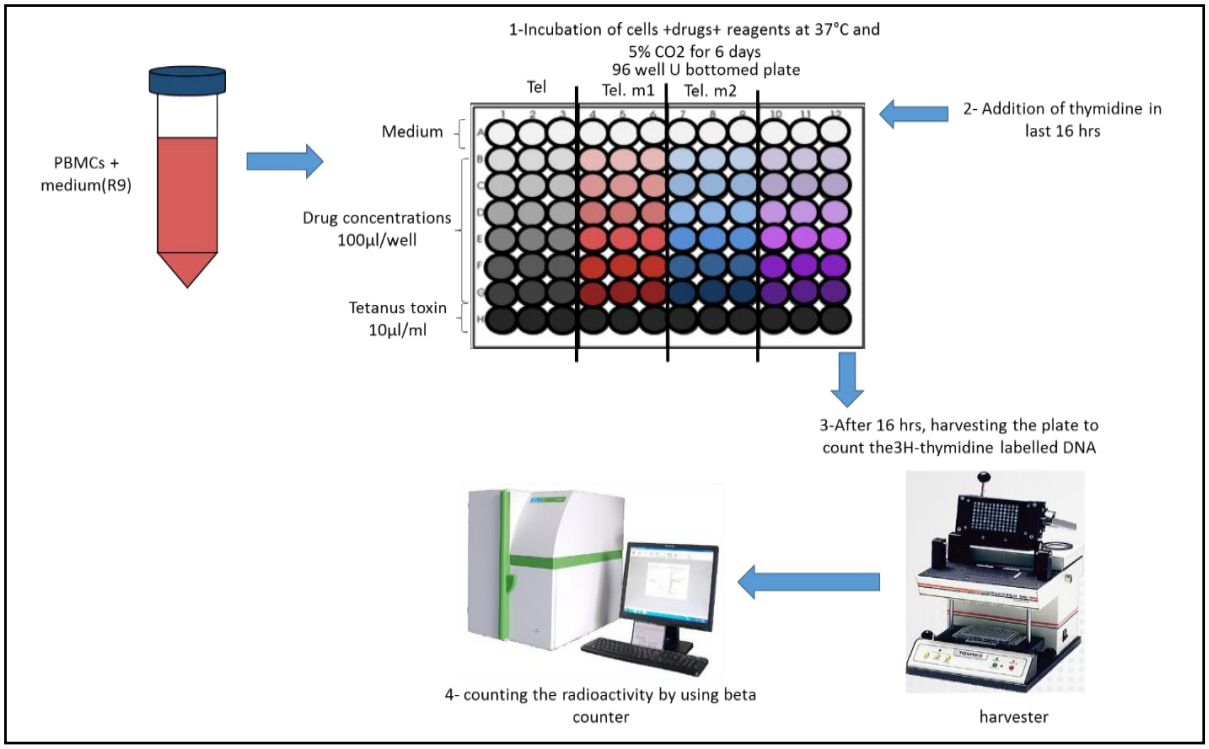


Figure 2-2 Lymphocyte transformation assay (LTT) scheme.

2.3.3 Generation of Epstein-Barr virus transformed B-cells.

Antigen presenting cell lines (EBVs) were generated from healthy donors or hypersensitive patients (Neitzel, 1986, Naisbitt et al., 2003, von Knebel Doeberitz et al., 1983).

Autologous EBVs are a fundamental requirement for the examination of T-cell clones. The marmoset cell line B9-58 acts as a source for the Epstein-Barr Virus (EBV), which is found

as circular episomal DNA inside the infected cells (5-800 copies/cell). This virus (EBV) selectively transforms human B-lymphocytes into an endlessly dividing, and immortalised lymphoblastoid cells (Neitzel, 1986).

PBMCs ($5x10^6$) either from patients or from normal volunteers were cultured with (5 mL) of supernatant from the B9-58 cell line filtered using 0.45 μm syringe filter. After that, cyclosporine A (CSA, 1μg/mL) was added to prevent the proliferation of T-lymphocytes, and PBMCs were incubated at 37°C overnight under an atmosphere of 5% CO_2. Cells were then spun at 1500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 2 mL of APC culture medium supplemented with (1 μg/mL) CSA. The cells were then transferred to new 24-well plates in a total volume 1 mL/well. Medium was replaced twice a week. CSA was stopped after a period of 3 weeks, and the cells transferred to cell culture flasks.

2.3.4 Generation of drug-specific T-cell lines and T-cell clones from PBMCs.

2.3.4.1 Drug-specific T-cell enrichment "bulk culture".

PMBC ($2x10^6$ cells/well) were cultured in 48 well plates with the culprit drug, in the optimal dose for activating T-cells for 14 days, at 37 °C and 5% CO_2. The cultures were fed on day 6 and 9 with culture medium and IL-2 (60 IU/mL).

2.3.4.2 Serial dilution of bulk cultures.

Bulk culture PBMC were diluted serially to 96-well U-bottom plates at 3 densities: 0.3, 1 and 3 cells/well, in culture medium supplemented with IL-2 (200 IU/mL), Phytohaemagglutinin (PHA) (5 μg/mL) and 45 Gy irradiated allogeneic PBMCs (as feeder cells). The serial dilution cultures were incubated for 2 weeks at 37 °C and 5% CO_2 with feeding every 48 hrs with culture medium (25 μL/well) containing IL-2 (60 IU/mL).

The restimulation cocktail was prepared with irradiated, allogeneic PBMCs at a concentration of $5x10^5$ cell/mL, IL-2 (400 IU/mL) and PHA (10 μg/mL). Serial dilution well volumes were reduced to 80 μL. Subsequently 50 μL was added to each well from the "restimulation cocktail". The cultures were incubated at 37 °C and 5% CO_2 for a further 14 days, during this period cells were fed every 2 days with culture medium (25 μL/well) supplemented with IL-2 (200 IU/mL), and the plates were regularly checked for any clone growth. Any wells that contained a mature clone, in the cell pellet (about 2mm) was transferred to new 96-U bottomed plate and expanded into 2 wells and later into 4 wells. When the clones were split into 4 wells, they were tested for drug-specific T-cell proliferation.

2.3.4.3 Proliferation of drug-specific T-cell clones using radioactive thymidine.

Half of each picked clone was split to 4 wells (50 μL/well) in 96-well plates and tested for antigen specificity. Autologous EBV-transformed B-cells were centrifuged to remove the F1 medium and resuspended in R9 medium. EBVs were irradiated for 20 min to stop cell proliferation but maintain their ability and process and present antigen. Irradiated EBVs were diluted to 10^4 cells/well and added (50 μL) to wells containing the clones. Two of the wells received 50 μL/well R9 medium as a negative control. The other two wells received 50 μL drug. Test plates were incubated for 48 hrs in a humidified atmosphere of 5% CO_2 at 37°C. [^{3}H]-thymidine (0.5 μCi) was added for the final 16 hrs. The tested clones were harvested on paper filter mats and read by the beta counter to measure the [^{3}H]-thymidine uptake by T-cell clones. The extent of the proliferation of the cells with drugs was divided by the proliferation of the cells in medium, to calculate the stimulation index (SI). If the SI was ≥ 2, it was accepted as a drug-specific T-cell.

2.3.4.4 Restimulation and expansion of drug-specific T-cell clones

After detecting drug-specific T-cell clones, the remaining 2 wells were transferred to sterile 3 mL tubes and centrifuged at 1500 rpm for 10 min at the room temperature. They were then resuspended in 330 μL R9 medium and added to one well of a 48-well culture plate. The restimulation cocktail consisting of irradiated allogeneic PBMCs which had been diluted to $1.5x10^6$/mL in R9 medium supplemented with IL-2 (400 IU/mL) and PHA (10 μg/mL) was then added to each well. Every two days clones were fed with 330 μL R9 medium supplemented with IL-2 (200 IU/mL).

2.3.5 Quantitative assessment of the clone responses to drugs (dose-response test).

The drug-specific T-cell clones were subjected to quantitative assessment of drug doses. Drug-specific T-cell clones were cultured with irradiated autologous EBV and serially diluted drug doses. A wide range of concentrations were selected around the optimum dose (higher and lower). Test plates were incubated for 48 hrs in a humidified atmosphere of 5% CO_2 at 37°C. [^{3}H]-thymidine (0.5 μCi) was added for the final 16 hrs. Tested clones were harvested on paper filter mats and read by the beta counter to measure the [^{3}H]-thymidine uptake.

2.3.6 Qualitative assessment of clone reactivity to drugs (cross-reactivity test).

The general protocol for T-cell proliferation by uptake of thymidine was also used here, but with this experiment T-cell clones were cultured with different concentrations of structurally related drugs.

2.3.7 T-cells proliferative response with and without APC fixation.

T-cell clones were harvested and suspended in HBSS (2 million/mL). Glutaraldehyde (1 μL /mL, 25%) was added to EBVs and the mixture was incubated at room temperature for 30s. The reaction was stopped by adding glycine (0.2M) for 45s. EBVs were washed with 3x25 mL medium. An additional set of EBVs (without fixation) was prepared, as a control for the experiment. Both types of EBV were irradiated and used in a [^{3}H]-thymidine uptake proliferation test (2.3.4.3).

2.3.8 APC pulsing assay.

Harvested EBVs were centrifuged to remove F1 medium and R9 medium was added. EBVs were incubated with the culprit drug for different periods with the same concentration of test drug in a 24 well plate for 1, 4, 16, 24 and 48 hrs. The time for pulsing was changed depending on the experiment. Harvested EBVs were washed, irradiated (60 Gy) and then incubated at (10000 cell/well/50 μL) in 96-well test plates. Other wells with non-pulsed irradiated (60 Gy) EBVs were added as controls. T-cells were prepared at a concentration of 50000 cells/well/50 μL. The drug was added in double effective concentrations. Control wells without the drug contained 100 μL medium. The plate was incubated for 48 hrs at 37°C and 5% CO_2. For the last 16 hrs [^{3}H]-thymidine (0.5 μCi)/well was added. The plate was harvested and read using a Beta counter (Figure 2.3).

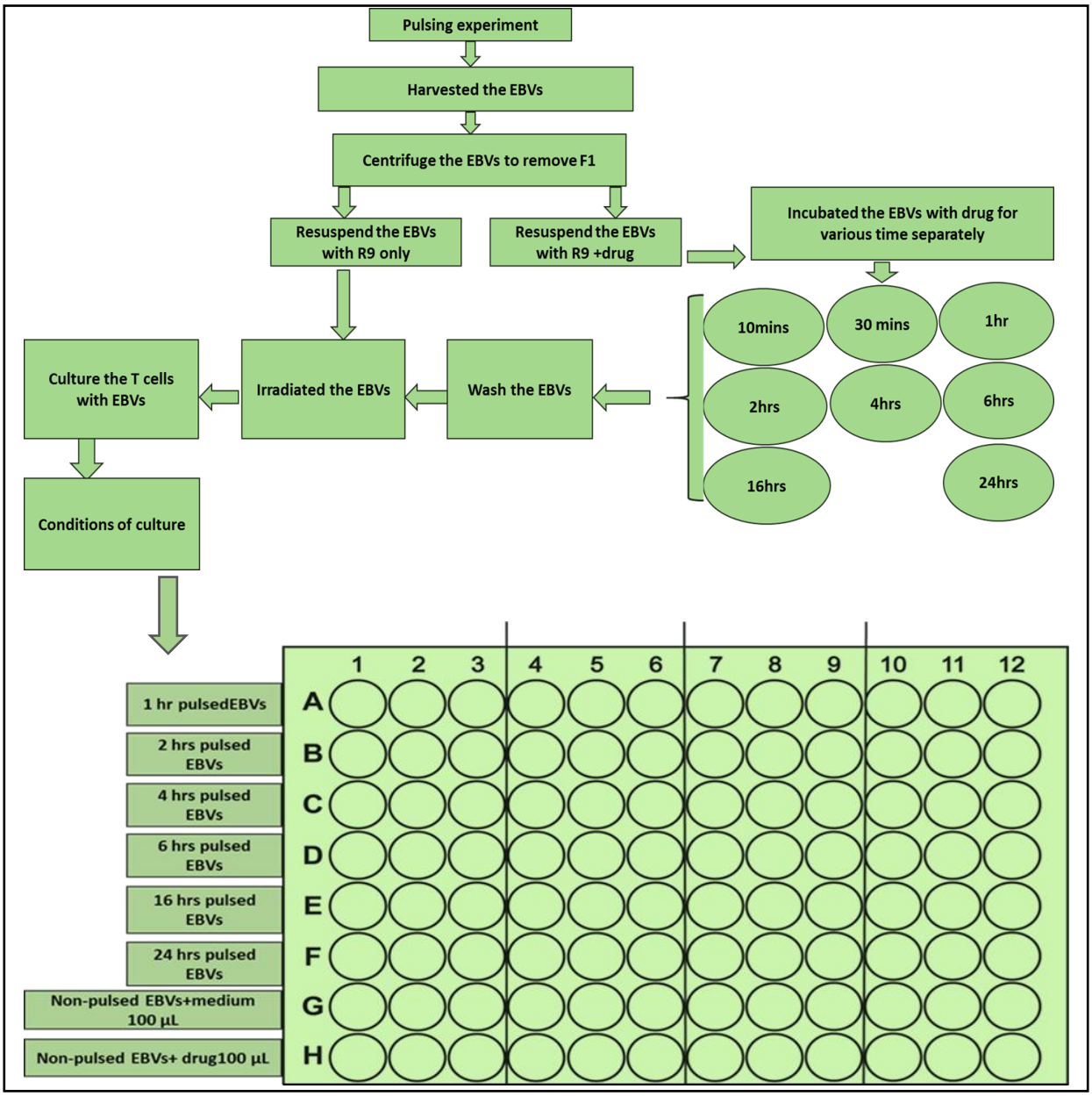


Figure 2-3 Pulsing assay. This assay was divided into two parts. First, pulsed EBVs were treated with the drug for different periods of time. Secondly, unpulsed EBVs were treated with soluble drugs. This group was considered as a positive control. The treated EBVs were washed several times to remove the unbound drug. Both groups (the treated and non-treated EBVs) underwent radiation.

2.3.9 Measurement of cytokine secretion from T-cell clones using Enzyme-linked immunospot assays (ELIspot).

This test is used to quantify individual cytokines-secreting cells when they encounter a specific antigen. There are several steps to the procedure (Figure 2.4). The limit of detection can be as low as 1 cell in 100,000 (Mabtech, 2018). ELIspots assays are carried out according to the manufacturer's instructions (Mabtech, Nacka Strand, Sweden).

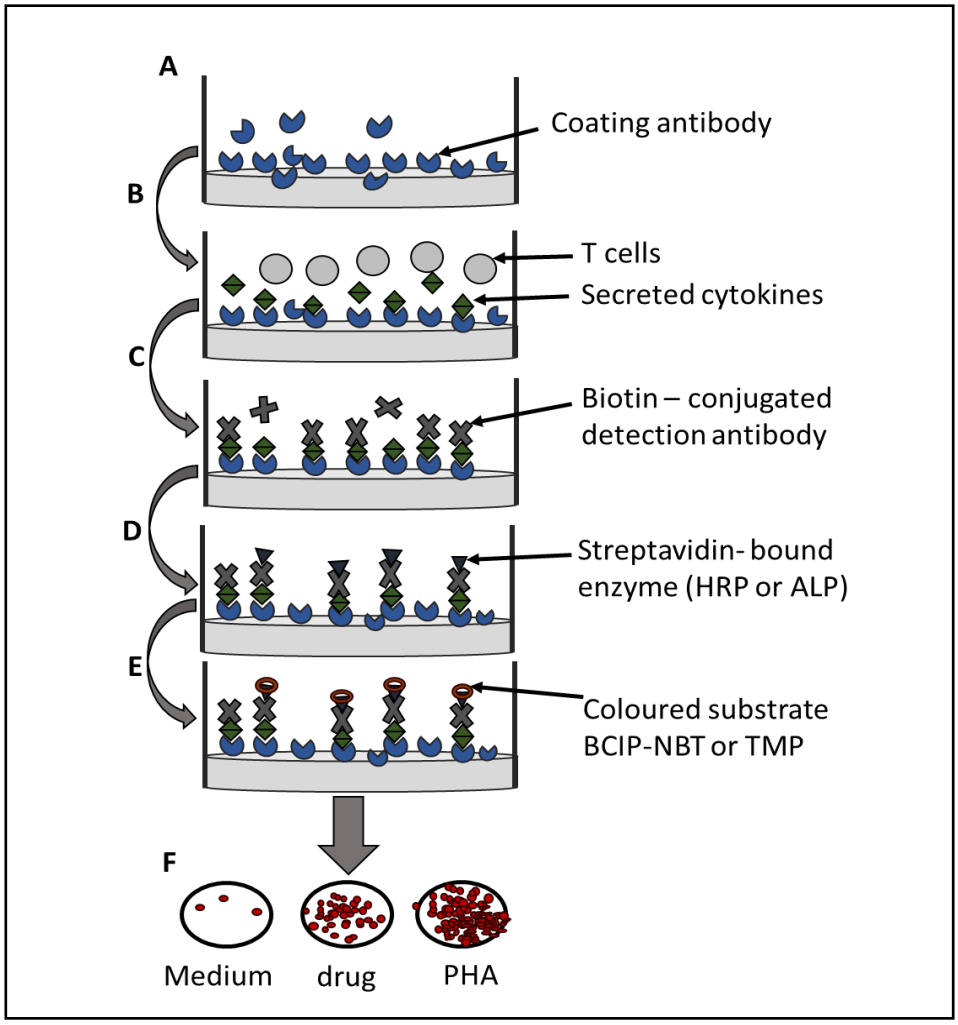


Figure 2-4 Detection of cytokine secretion using ELIspot. A. The ELIspot plate is coated with cytokine-specific coating antibody and is incubated overnight at 4°C. B. The plate was washed and the membrane was blocked with R9 medium. T-cells, APCs and drug were added and incubated for 48 hrs at 37°C. C, The plate was washed, and biotin-conjugated detection antibody was added and incubated at room temperature for 2 hrs. D. The plate was washed, streptavidin-bound enzyme (HRP or ALP) was added and incubated for 1 hr at room temperature. E. The plate was washed and substrate was added (BCIP-NBT or TMB). Spots start to appear during 15 min incubation in the dark. (F) The plate was washed and left to dry overnight, to be ready for reading by the ELIspot reader.

A. Coating the ELIspot plate: On day 0.

Ethanol (20 µL, 35%) was added to each well of the ELIspot plate and left for one minute. The plate was washed 5x with sterile dH_2O (200 µL/well). Coating antibody (100 µL/well) was added and incubated overnight at 4°C. The antibodies were diluted from the stock (IFN-γ, diluted from 1 mg/mL to 15 µg/mL; IL-13, IL-22, IL-17, diluted from 0.5 mg/mL to 10 µg/mL) in HBSS. 100 µL of coating antibody was added to each well and then incubated overnight at 4°C.

B. Cell Incubation: On day 1.

The coated plate was washed 5x using 200 µL HBSS, to remove the excess antibody. The plate was blocked by adding culture medium (200 µL/well) for 30 min. After washing 5x with HBSS, $5x10^4$/50 µL T-cells, $1x10^4$ 60 Gy irradiated EBVs/50 µL, medium (negative control), serial dilutions of culprit drug 100 µL/well, or PHA (5µg/µL) as a positive control, were added to the wells. The plate was wrapped in aluminium foil and incubated at 37 °C and 5% CO_2 for 48 hrs.

C. Spot Detection: On day 4.

On completion of the incubation the plate was washed 5x with 200 µL PBS. Detection antibody (100 µL/well) was added and incubated for 2 hrs at room temperature. The plate was washed 5x with 200 µL PBS. Streptavidin ALP (100 µL/well) was added and incubated for 1 hr at room temperature. The plate was washed 5x with 200 µL PBS and afterwards 100 µL/well of detection substrate (BCIP/NBT) was added. The plate was kept in the dark at room temperature. The final step was waiting until the spots emerged (generally they appeared in 13-15 min). The reaction was stopped by washing the plate with tap water and then the plate was rinsed and kept in dark until it dried. The plate was then analysed using an AID ELIspot reader (Cadima Madical, Stourbridge, UK) to count the spots.

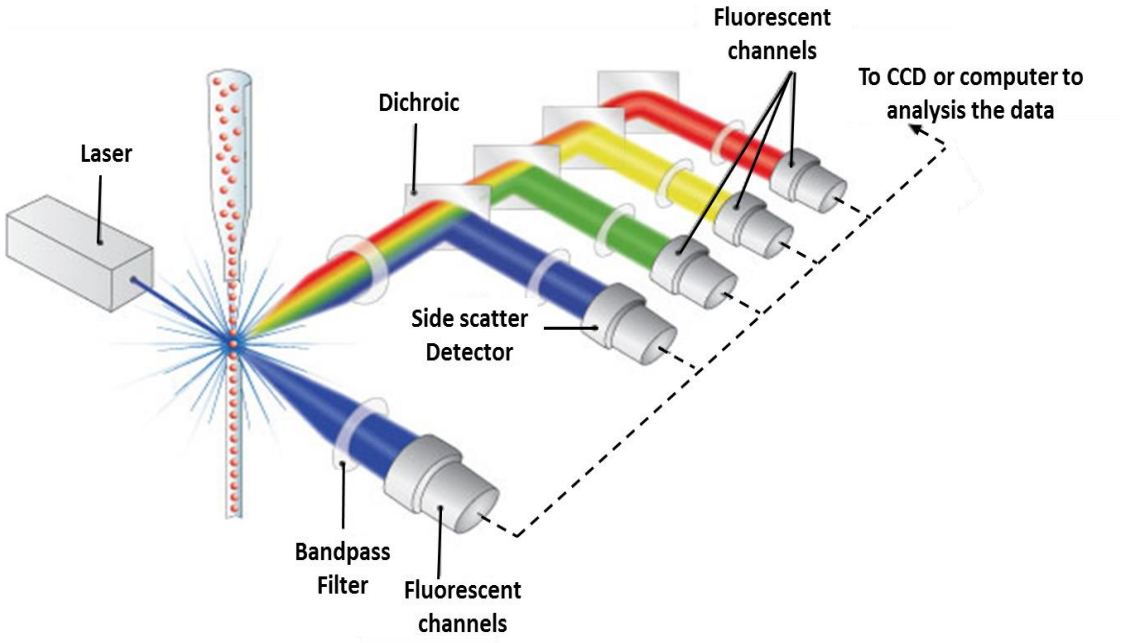
2.3.10 MHC restriction assay.

MHC restriction assay was conducting using two methods, either by assessment proliferation or by ELIspot assay to detect cytokine secretion from T-cell clones. The method follows the previous protocols for proliferation and cytokine release. Autologous EBV-transformed B-cell lines ($1x10^4$, 50 μL) were incubated with either MHC I or MHC II blocking antibodies at a concentration 5 μg/mL in a humidified atmosphere of 5% CO_2 at 37°C for 30 min. The EBVs were then cultured with drug-specific T-cell clones ($5x10^4$, 50 μL) with or without a drug for 48 hrs. After the incubation period, [^{3}H]-thymidine (0.5 μCi) was added for the final 16 hrs and plates were harvested onto filter mats using a TomTec Harvester 96. Incorporated radioactivity was measured as described previously. Cytokine secretion profiles (IFN-γ, IL-5, IL-13and granzyme-B) were determined by using ELIspot analysis using the procedure described on the previous page.

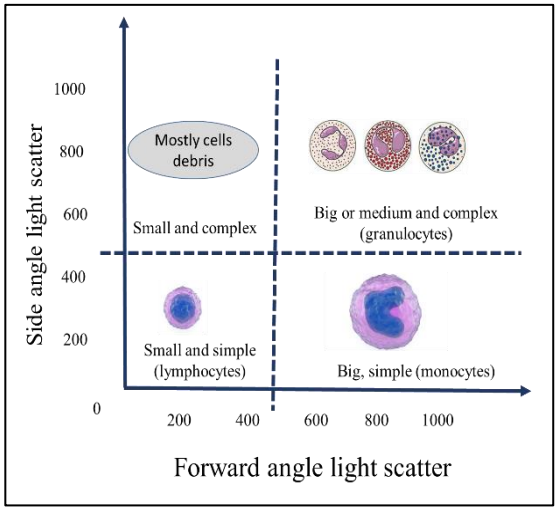
2.3.11 Measurement of T-cell receptor and chemokine receptor expression using flow cytometry.

Flow cytometry was created in the 1970s. It became a very important method for detection of the specific markers on the surface of cells and to measure single cell characteristics. Flow cytometry depends on the suspension of cells in a narrow liquid flow so that they pass in a single file through the path of multiple laser beams, with different wavelengths. Optical detectors transform fluorescent light emitted from each cell into an electrical signal (Figure 2.5 A). Most of the cells are labelled with fluorescent antibodies that bind to specific membrane proteins. Depending on the density of signal emitted at various wavelengths, the cells are analysed one by one for various features, for example, size, granularity, and expression of membrane-bound proteins. By this method, thousands of single cells can be analysed per hour, for up to 18 protein markers (Figure 2.5 B, C) (Nassar et al., 2015).

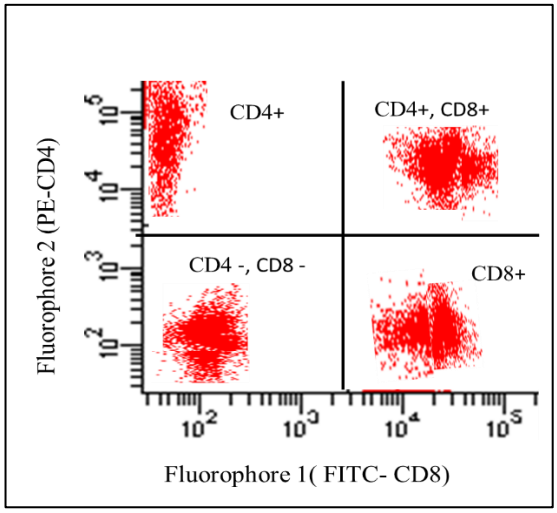
A. Flow cytometry technique.



B. Data analysis for types the cells.



C. Phenotyping for type cells.



D. The T-cells gate

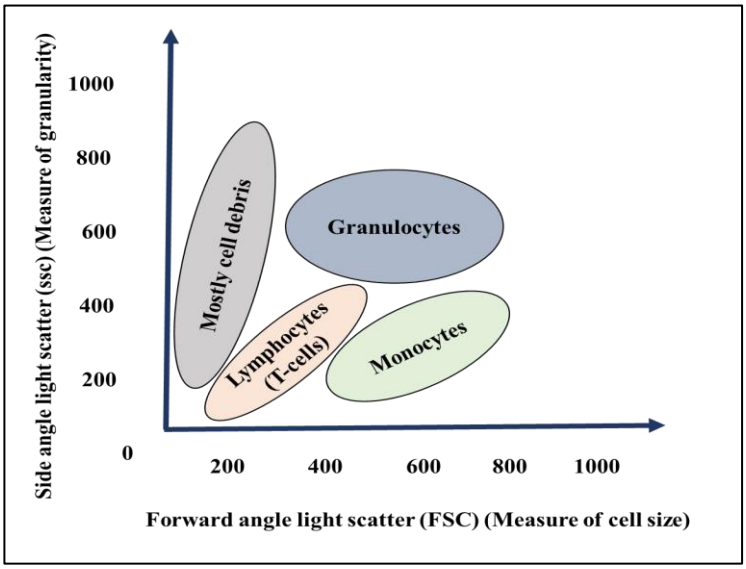


Figure 2-5 A. The principle of phenotyping cells using flow cytometry. The figure is adapted from (Semrock, 2018).The cell populations are stained with fluorochrome-bound antibodies. To detect multiple markers, the individual fluorochromes must be associated with various wavelengths to help distinguishing them. After passing the cells through the flow cell tube as a single cell stream in fast flowing sheath fluid (hydrodynamic focusing), each individual cell will pass through the channel of the laser light. Fluorochromes bound to markers on the cell surface diffuse light at their particular wavelengths, and this will be detected with sensor specific for different wave-bands that have the ability to detect the emitted light. B. Data analysis depends on the differential refraction of the light based upon the cell size and granularity that is detected by the side scatter (SSC) - and forward scatter (FSC)-sensors to provide information indicative of particular cell populations. If the cells appear in the upper half that means the cells give high signals at the side scatter detectors, which means that cells are complex. If the cells appear in the left side that means the cells give low signals of forward light scatter, which means that cells are small. If the cells appear in the lower quarter that means the cells give low signals at the side scatter detectors, which means that the cells are simple. If the cells appear in the right half that means the cells give high signals of forward light scatter, which means that cells are large. C. Phenotyping. This gated population is analyses of the type of the cells depending on various kinds of specific proteins on the cell surface by using two different fluorescent stains such as CD4, CD8. D. The T-cell population (lymphocytes) is gated on a dot plot of FSC and SSC.

The protocol below describes a general method for flow cytometric analysis.

Compensation Preparation: Due to the fact that this experiment contains multicolour staining, compensation should be performed. The compensation is used to prevent

interference of the fluorescent dyes signals. The compensation kit consists of 2 droppers, they contain beads which can be stained with the fluorochrome antibodies used in the analysis. The first step is that the FACS buffer (400 µL), is added to one non-sterile FACS tube. Then, one drop of each compensation bead is added to the tube. After shaking, (100 µL) of the mixture is transferred to non-sterile FACS tubes. The number of compensation tubes equals the number of the colours of fluorescent antibodies used in the experiment, plus one extra tube for the control condition.

Cell labelling: 50 µL of cells were transferred into a non-sterile FACS tube. The fluorescent stain bound antibodies were added into the FACS tube at a concentration determined by the manufacturer. Then, the tubes were incubated at 4°C for 20 min in the dark. After the incubation period, 500 µL of FACS buffer was added to each tube. Subsequently, they were centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the cells were resuspended in 200 µL FACS buffer for immediate analysis by flow cytometry. In case of delayed the analysis, the cells were fixed in 4% paraformaldehyde (200 µL) and kept in the fridge at 4ºC.

2.3.12 Migration of T-cell clones.

A Corning Costar Transwell plate containing 12 wells and a pore size of 5µm was prepared by adding medium (3 mL) with chemokines (48 µL) to the lower chamber of the well. Specifically, CCL17, CCL27 and a mixture of CCL17 and CCL27 were used to identify the migration of drug-specific T-cell clones. Clones were added to the top chamber and their migration was assessed by counting the increase in number of cells in the bottom compartment at different time points (30 min, 2 hrs, 4 hrs, 16 hrs, and 24 hrs).

2.3.13 Isolation of cell subsets from whole PBMC using MACS magnetic bead columns and the priming of naïve T-cells from healthy donors.

In this experiment different populations of cells were separated from total PBMCs using magnetic beads and columns in a sequence steps according to the manufacturers' instructions (Miltenyi Biotec, Bisley, UK). The purified cells were utilized to perform tests which needed pure populations of $CD14^+$ monocytes, $CD45RO^-$ $CD45RA^+$ (naïve) T-cells, and $CD45RO^+$ $CD45RA^-$ (memory) T-cells. During **positive selection**, antibodies specific for the required cell surface marker protiens were bound to 50 nm diameter, non-toxic, biodegradable, superparamagnetic particles to produce MACS microbeads. These MACS microbeads bind to the cell surface. The labelled cells could be easily separated from non-labelled cells by passing the total cell population through a magnetic field. The microbeads do not block all the epitope on each cell, thus allowing cells to maintain their functionality. The labelled cells bound to the magnetic beads are retained within the column. After that, the magnet is removed and the labelled cells are pushed out of the column by plunging the column with MACS buffer. The second type of selection is **negative selection** attributed to the use of the antibodies which select markers that are not found on the cell population of interest. Selections yield population purities of > 97% (Faulkner et al., 2012b).

Procedure: A sample of blood would be taken from a volunteer and the PBMCs isolated.

2.3.13.1 Positive selection of $CD14^+$cells monocytes.

All cells, microbeads and MACS buffer were kept on ice. PBMCs were centrifuged for 10 min at 1500 rpm. The supernatant was removed, and the cells were resuspended in MACS buffer (800 μL per 10^8 PBMCs). $CD14^+$ specific microbeads (200 μL per 10^8 cells) were added, then the cells were incubated at 4ºC for 15 min. 15 mL MACS buffer per 10^8 cells was added to the cells suspension, which was then centrifuged for 10 min, 1500 rpm at 4ºC.

The supernatant was removed and the cells resuspended in MACS buffer (500 µL per 10^8 cells). A MACS LS column was added to the magnet and washed with MACS buffer, then the cell suspension was added directly to column. The column was washed 3x with 3 mL MACS buffer to ensure all unlabelled cells had passed through the column and been eluted. The collection tube contains the $CD14^-$ cell population, while the $CD14^+$ population stick to the column. The column as then removed from the magnet, and placed over a new collection tube to collect the cells from the column by plunging 5 mL MACS buffer firmly through the column into a new collection tube. These were the $CD14^+$ cells (Figure 2.6 A). MACS buffer (10 mL) then was added to the $CD14^+$ and $CD14^-$ cells. Both of cells were counted, centrifuged and then resuspended in R9 medium. 500 µL of the cell suspension was taken to generate dendritic cells. The remaining cells were cryopreserved at 5-10x10^6 cells/cryovial.

2.3.13.2 Negative selection of $CD3^+$ T-cells.

Further isolation was performed using the total T-cell population (Figure 2.6 B). Non-CD14 cells were spun down and the supernatant was discarded, then they were resuspended in MACS buffer (400 µL per 10^8 cells). A pan T-cell antibody cocktail (100 µL per 10^8 cells) was added. This cocktail consisted of different types of antibodies prepared for selective markers that would not be found on T-cells to allow for later positive selection of these cells and for T-cells to pass through the column. The cells were mixed and incubated at 4°C for 10 min. After the first incubation period was finished, more MACS buffer (300 µL per 10^8 cells) was added as well as anti-biotin microbeads (200 µL per 10^8 cells). The cells were entered to a second incubation period at 4ºC for 15 min. Next, the cells were diluted in MACS buffer (1.5 mL per 10^7cells). Then they were centrifuged at 1500 rpm for 10 min at 4°C. The supernatant was removed and resuspended in the MACS buffer (500 µL per 10^8 cells) and passed through a new LS column as previously described in positive selection of $CD14^+$ cells. The cells that travel into the collection tube were $CD3^+$ cells. The volume of T-cells

($CD3^+$) was increased to 15 mL. The non-T-cells ($CD3^-$) were also collected from the column. Both cell subsets were counted before centrifuging. Non-T-cells ($CD3^-$) were frozen and stored for use in the setup of Epstein-Barr virus transformed B-cells (EBV) as discussed in section 2.3.3.

2.3.13.3 Positive selection of $CD25^+$ (regulatory) and $CD45RO^+$ (memory) T-cell populations

The T-cell population ($CD3^+$) was then subject to two final positive selection processes. First, T-cells were resuspended in MACS buffer at a concentration of (900 μL per 10^8 cells) and $CD25^+$ microbeads (100 μL per 10^8 cells). The cells were then incubated for 15 min at 10ºC. After centrifugation the cells were resuspended in MACS buffer (500 μL per 10^8 cells), and pushed through a new LS column as previously described. The $CD25^-$ T-cells crossed the column to the collection tube. While the $CD25^+$ T-cells were retained in the column. $CD25^+$ T-cells were subsequently extracted from the column. In the second selection, the $CD25^-$ T-cells were resuspended in MACS buffer (800 μL per 10^8 cells) and CD45RO microbeads (200 μL per 10^8 cells), and the mixture was incubated for 15 min at 4ºC. After washing, the cells were resuspended in MACS buffer (500 μL per 10^8 cells), and passed through a new LS column as previously described. The $CD45RO^-$ T-cells passed directly through the column, while the $CD45RO^+$ T-cells were retained. The $CD45RO^+$ T-cells were subsequently eluted by force. The final step was that all the cells ($CD25^+$ T regulatory cells, $CD45RO^+$ memory T-cells, $CD45RO^-$ naïve T-cells), were counted, centrifuged and then frozen at a concentration of 5-10x10^6 cells per cryovial in a 1:1 mixture with "freezing mix".

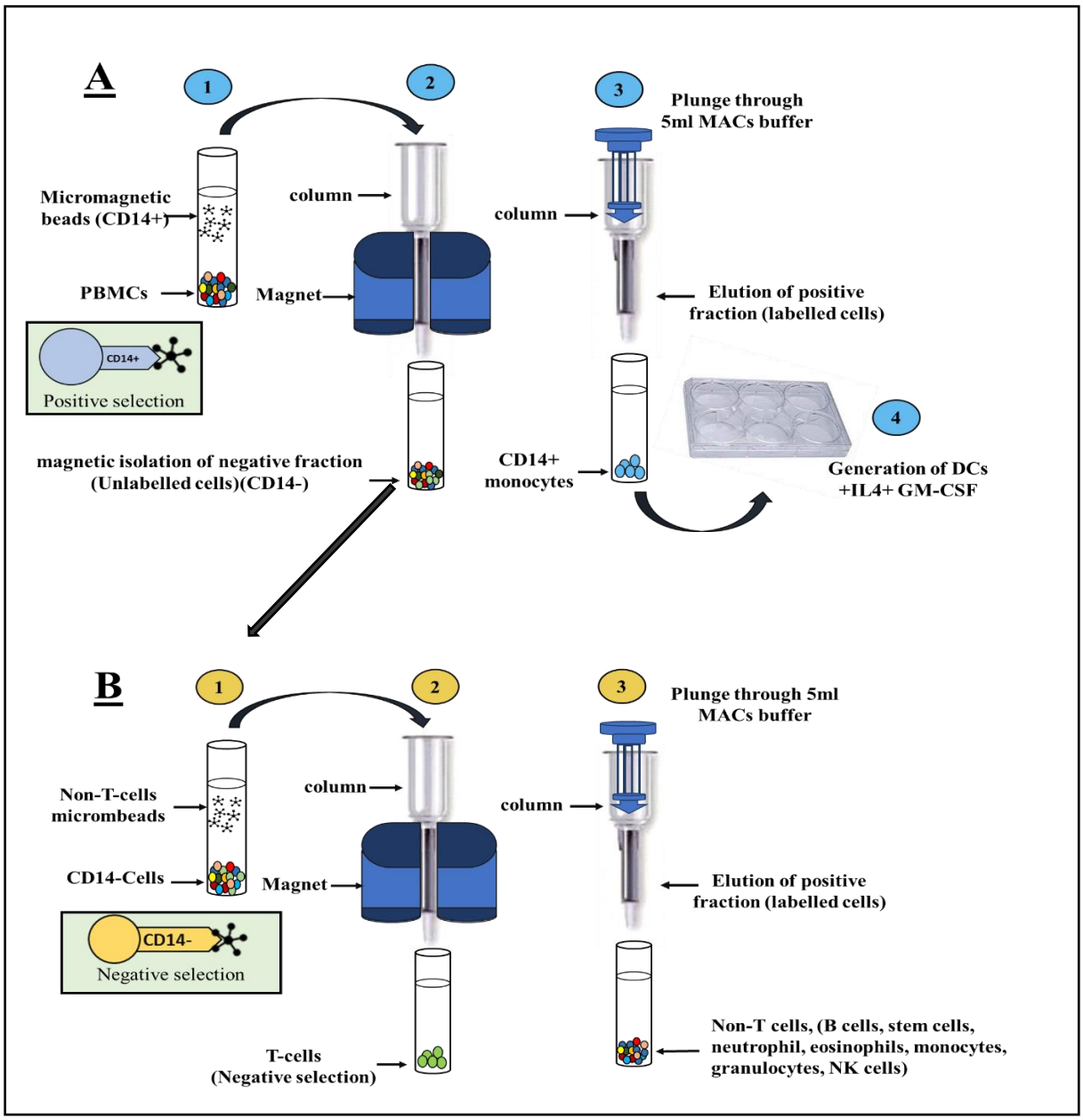


Figure 2-6 Magnetic bead-mediated isolation of various cell subsets from healthy donor PBMCs. (A) the first step was to add CD14 microbeads. The labelled PBMCs were then added to a column placed on a magnet. The $CD14^+$ labelled cells stick in the column, whereas, the $CD14^-$ would wash through. Then the column was removed from the magnet and the $CD14^+$ cells eluted. (B) Next, the $CD14^-$ cells were incubated with a biotinylated antibody cocktail which connects to a range of cell surface molecules not associated with T-cells. After that, anti-biotin microbeads were added to bind to these antibodies. When the cells were added to the column, T-cells were washed through. The non-T cells still binding in the column, therefore would be forcibly eluted from the column. Other steps for further separation, (C) First isolation of $CD25^+$ cells by positive selection (Treg) (D) Second, separation were then performed of $CD45RO^+$ cells from the $CD25^-$ cells was conducted. Finally, naïve ($CD45RO^-$) T-cells were separated from their $CD45RO^+$ (memory) T-counterparts. (C and D not found in the graph).

2.3.14 Generation of dendritic cells from $CD14^+$ monocytes.

$CD14^+$cells (monocytes) can differentiate into dendritic cells (DCs). DCs act as APCs and are effective at priming of naïve T-cells (Guermonprez et al., 2002).

To generate DCs, frozen $CD14^+$ monocytes were thawed then transfered to a fresh tube of R9 medium. The thawed cells were centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the cells resuspended in R9 medium for counting. The $CD14^+$ cells were suspended in medium containing GM-CSF (800 IU/mL) and of IL-4 (800 IU/mL) and aliquoted into a 6 well plate. The cells were then incubated overnight in a humidified atmosphere containing 5% CO_2 at 37°C (Faulkner et al., 2012a). 3 mL of the medium from each well (supernatant) was withdrawn and discarded. Then, 3 mL of the medium containing GM-CSF (800 IU/mL) and IL-4 (800 IU/mL) was added. The cells were fed as in day 4 the penultimate day before use on but with the addition of the maturation factors LPS (1 μg/mL; bacterial source reference, Escherichia Coli 0111:B4) and TNF-α (25 ng/mL).

2.3.15 Activation of naïve T-cells with drugs.

Activation of naïve and memory T-cells was studies in vitro (Figure 2.7). Autologous mature DCs were harvested from 6 wells plate, centrifuged and counted. The DCs were plated at a concentration of $0.8x10^5$ per well, and cultured with naïve or memory $CD3^+$ T-cells at a concentration of $2.5x10^6$ per well; 24-well flat-bottomed plate. The volume in the wells was 2 mL (Figure 2.7 F). The drugs and metabolites were prepared at on optimum doses and added the cells. The cells were incubated in conditions of 5% CO_2 at 37°C for 8 days. During incubation period a second batch of mature DCs were generated from autologous $CD14^+$ monocytes.

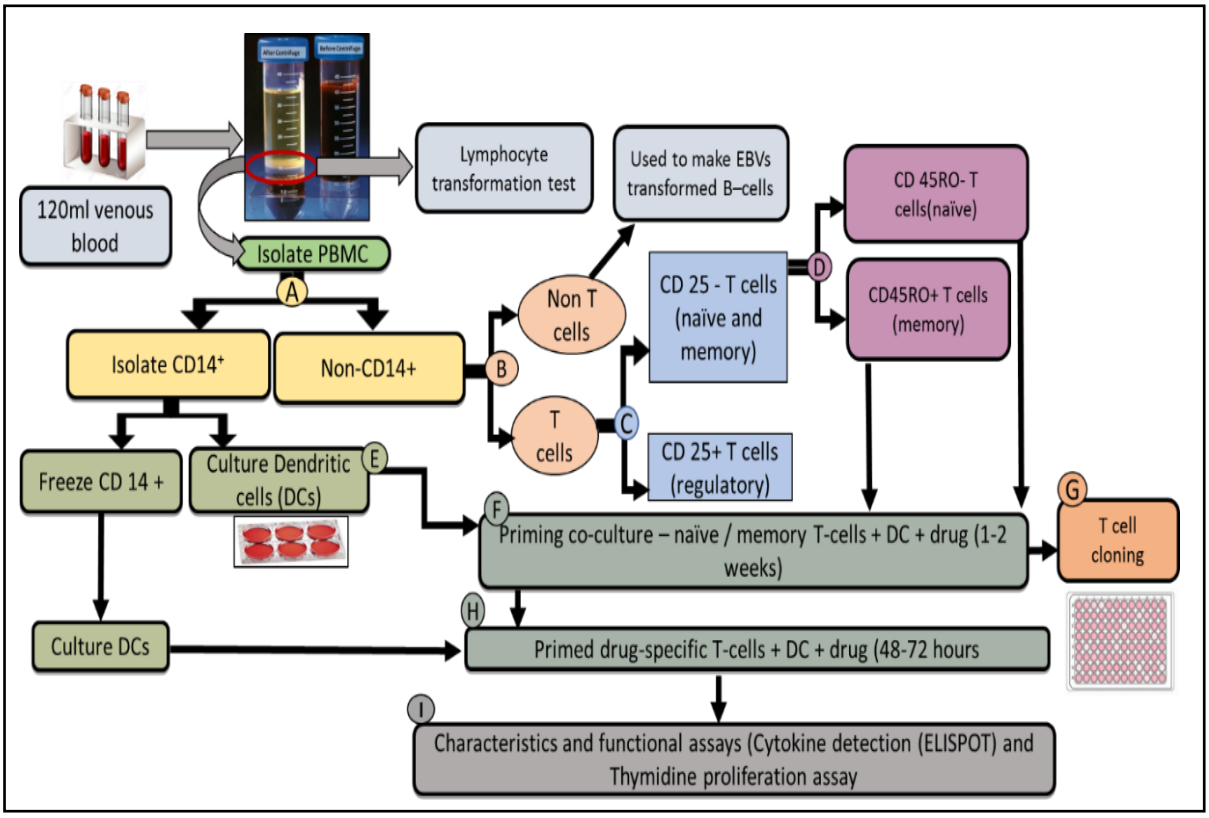


Figure 2-7 T-cell activation assay (in vitro). Isolation of PBMCs from venous blood. A: first isolation for $CD14^+$ cells. B: isolation the $CD3^+$ T-cells by negative selection from $CD14^-$ population. C: isolation the types of T cells ($CD25^+$ and CD25- T-cells). D: CD 25- isolated more to $CD45RO^+$ cells (memory) and $CD45RO^-$ T-cells (naïve) and $CD45RO^+$ T-cells (memory). E: culture the $CD14^+$ monocytes to generate DC and then matured. F: Naïve or memory T-cells are cultured with mature DC and drug. G: after 1 week of culture T-cells were harvested and used for T-cell cloning, or H: collected with fresh mature DC and drug to study the features of T-cells. I: types of assays.

2.3.16 Reactivity and specificity assays for T-cells.

The aim of these experiments is to detect antigen-specific T-cell responses generated from naïve and memory T-cell cultures.

A. [3H]-Thymidine incorporation to measure antigen-specific T-cell proliferation.

Priming cultures were harvested, centrifuged, resuspended in R9 medium and counted. The desired concentration for the primed cells $1x10^6$/mL. Mature DCs were harvested by cell scraping. The DCs were centrifuged, resuspended in R9 medium, counted and diluted to a

cell concentration of $0.8x10^5$/mL (Figure 2.7 H). Primed T-cells ($1x10^5$/well) were cultured in 96 well test plates (100 μL) along with $4x10^3$ mature DCs (50 μL) in triplicate cultures per condition. The drugs were prepared in non-toxic doses, alongside a positive (Phytohemagglutinin (PHA) 20 μg/mL) and negative control (R9 medium). The test plate was incubated in a humidified of 5% CO_2 at 37°C for 48 hrs. [^{3}H]-thymidine (0.5 μCi/well) was added in last 16 hrs. The test plate was harvested onto filter mats and dried. The filter mats were then combined with hot wax and counted in a Micro Beta counter. After counting the ratio of proliferation compare to controls conditions was calculated. A stimulation index (SI; average cpm of drug-treated wells/average cpm of control wells) of ≥1.5 was accepted as a drug-specific T-cell response.

B. IFN-γ, IL-13, IL-17, and IL-22 secretion to measure antigen-specific T-cell response.

The ELIspot plate was coated prior to setting up the T-cell assays. Antibodies for IFN-γ, IL-5 and IL-13 were used according to the procedure was described in section 2.3.9. The next day, primed T-cells ($1x10^5$; 100 μL) and $4x10^3$ mature DCs (50 μL) were added to the plate. The drug was added in 50 μL R9 media alongside 50 μL R9 and PHA (20 μg/mL) as negative and positive controls, respectively. The cells were incubated in a humidified of 5% CO_2 at 37°C for 48 hrs. The ELIspot plates were washed and read using the procedure was described in section 2.3.9.

2.3.17 Assessment of DC and T-cell phenotype using flow cytometry.

The T-cell surface markers were detected during priming and following restimulation using the method discussed in section 2.3.11, to determine whether the primed cells are either $CD4^+$ or $CD8^+$. Furthermore, to detect the phenotype of the monocyte-derived mature DCs various antibodies including CD1a-FITC, CD11a-FITC, CD11c-PE, CD14-FITC, CD40-FITC, CD80-FITC, CD83-PE and CD86-FITC were used. The cells were incubated with

antibodies in the dark for 20 min, washed with 1 mL FACS buffer, centrifuged (1500 rpm for 10 min at 4ºC), resuspended in 200 μL FACS buffer, then analysed using a FACS-Canto II flow cytometer.

Chapter 3: Dapsone and Nitroso dapsone-specific activation of T-cells from hypersensitive patients expressing risk alleles HLA-B*13:01.

3.1 Introduction

Genome-wide association studies have identified strong associations between expression of single HLA alleles and susceptibility to different forms of drug hypersensitivity (Redwood et al., 2018). These ground-breaking data propose that drugs might bind selectively to the protein encoded by the HLA allele to activate the T-cells that participate in the adverse event. Molecular docking studies seem to support this concept, drugs can interact directly with T-cells and HLA proteins (Teh et al., 2016, Van Den Driessche and Fourches, 2017). However, modelling data has to be interpreted with caution as the nature of the drug that interacts with the HLA protein and the requirement for a specific peptide in the binding groove has not been determined. The most robust genetic associations are between HLA class I alleles and abacavir hypersensitivity (Mallal et al., 2008), flucloxacillin liver injury (both HLA-B*57:01) (Daly et al., 2009), carbamazepine-induced Stevens-Johnson syndrome (HLA-B*15:02) (Chung et al., 2004b) and allopurinol hypersensitivity (HLA-B*58:01) (Hung et al., 2005). All mechanistic studies in these cases have shown that $CD8^+$ T-cells are stimulated during the interaction between the drug and the relevant HLA protein (Monshi et al., 2013b, Yun et al., 2014, Illing et al., 2012, Ostrov et al., 2012, Ko et al., 2011). There are differences in the drug HLA binding such as for abacavir and carbamazepine, abacavir binds deep in the HLA peptide binding pocket, while carbamazepine binds to a site closer to the T-cell receptor interface. Despite this, both drugs interact with HLA proteins via a reversible interaction to stimulate T-cells. T-cells from patients with allopurinol hypersensitivity are activated with a stable metabolite, oxypurinol, via direct binding interaction with HLA. In contrast, flucloxacillin-specific T-cells from patients with liver injury are activated with drug-protein adducts, via a hapten mechanism involving protein processing. This brief discussion illustrates that rapid progress has been made in our understanding of the relationship between drug HLA binding and the activation of T-cells; however, reactive drug metabolites have

been ignored. This is primarily because of the absence of synthetic reactive metabolites for drugs associated with HLA allele-restricted forms of hypersensitivity. Recently, we synthesized the nitroso metabolite of dapsone and studied the priming of naïve T-cells from healthy donors (Alzahrani et al., 2017b). The chemical structure for dapsone (DDS) contains a sulfone group that links two aromatic amine moieties. Oxidative metabolism of the amine groups generates a hydroxylamine intermediate. The hydroxylamine undergoes spontaneous oxidation to form nitroso dapsone (DDS-NO), which binds covalently to dendritic cells and keratinocytes (Roychowdhury et al., 2007, Vyas et al., 2006a). Naïve $CD4^+$ and $CD8^+$ T-cells which were generated from healthy donors expressing different HLA-B alleles were activated with the parent drug and nitroso metabolite when (1) Tregs were removed and (2) the compounds were presented to naïve T-cells by dendritic cells (Alzahrani et al., 2017b). These data confirm that the drug in both forms interacts with multiple HLA molecules and has the capacity to stimulate T-cells when regulatory pathways have been manipulated.

DDS is used in combination with other drugs for the treatment of various infectious diseases such as leprosy and malaria. Between 0.5-3.6% of treated patients develop a hypersensitivity syndrome characterized by fever, skin rash and internal organ involvement 4-6 weeks after treatment commences (Rao and Lakshmi, 2001). The culprit allele HLA-B*13:01, is associated with the development of DDS hypersensitivity in Chinese and Thai patients (Zhang et al., 2013, Tempark et al., 2017), and modelling data suggests that DDS may fit in the peptide recognition site of HLA-B*13:01 (Watanabe et al., 2017). DDS-treated cell lines expressing HLA-B*13:01 appeared to stimulate T-cells, while PBMC from 2/7 patients appeared to secrete high levels of the cytolytic molecule granulysin when stimulated with the drug (Chen et al., 2018).

Despite all these studies, a detailed analysis of the phenotype and function of DDS-specific T-cells from hypersensitive patients has not been performed. Moreover, the activation and generation of patient T-cells with DDS-NO has not been investigated. Thus, our study had three primary objectives: (1) to investigate whether DDS and/or DDS-NO activates $CD4^+$ and $CD8^+$ T-cells from hypersensitive patients; (2) to define the phenotype and function of drug-specific T-cells and (3) to explore whether HLA-B*13:01 is directly involved in the drug- / drug metabolite-specific T-cell response.

3.2 Aims of the study.

- Generation and characterization of DDS and DDS-NO-specific $CD4^+$, $CD8^+$ T-cell clones from patients with DDS hypersensitivity.
- To investigate the individual roles of DDS and DDS-NO in activation the drug-specific T-cell clones.
- To explore the mechanisms of T-cell activation.
- To determine the role of HLA-B*13:01 in the activation of DDS- and DDS-NO-$CD8^+$ T-cell clones.
- To investigate the function, reaction and phenotypic characteristics of specific T-cells clone.

3.3 Methods.

The methods used in this chapter are discussed in detail in chapter 2 with some changes in some materials to be suitable for DDS and DDS-NO study.

3.3.1 Study approval.

The study was approved by the Ethical Committee of the Shandong Provincial Institute of Dermatology and Venereology and informed written consent was received from participants

prior to inclusion in the study. A material transfer agreement was signed prior to shipment of cryopreserved PBMC to Liverpool.

3.3.2 Human subjects and cell isolation/separation.

In this study we used venous blood (50 mL) collected from six DDS hypersensitive patients. Table 3.1 summarizes the demographics of the patients and details the nature of the adverse events. Table 3.2 shows the results of HLA typing. HLA-B*13:01+ donors (n=4) with no history of DDS exposure were selected as a control group. PBMC were isolated and used for the lymphocyte transformation test, IFN-γ PBMC ELIspot, the generation of EBV-transformed B-cells and T-cell cloning. Patch testing was conducted on the back of hypersensitive patients with DDS concentrations between 0.1-25%. The patch was removed after 48 hrs and the results were recorded 24 hrs later.

Table 3-1 Patient demographics and details of the hypersensitivity reaction.

Patient ID	**Gender**	**Age (years)**	**Medication history**	**Onset of symptoms (days)**	**Clinical presentation**	**Skin patch test**
1	Female	43	Dapsone, rifampin, and clofazimine	3	Fever, rash, and abnormal liver function tests	-
3	Female	25	Dapsone, rifampin, and clofazimine	28	Fever, rash, and abnormal liver function tests	-
5	Male	39	Dapsone, rifampin, and clofazimine	30	Fever	+
6	Male	41	Dapsone, rifampin, and clofazimine.	48	Fever, rash, and lymphadenopathy	+
7	Male	54	Dapsone, rifampin, and clofazimine	16	Fever, rash, and abnormal liver function tests (AST 47.7 U/L; ALP132.3 U/L)	-
8	Female	27	Dapsone, rifampin, and clofazimine	17	Fever and abnormal liver function tests (AST 56.3 U/L; ALP 225.7 U/L; GGT 184.8 U/L; TBIL 38.8 umol/L; DBIL 21.3 umol/L; IBIL 17.5)	+

Table 3-2 HLA typing of hypersensitive patients.

	HLA-A		**HLA-B**		**HLA-C**		**HLA-DQB1**		**HLA-DRB1**	
Patient 1	02:07	24:02	*13:01*	40:01	03:04	15:02	03:01	06:01	11:01	15:01
Patient 5	02:07	11:01	*13:01*	40:01	03:04	07:02	06:01	06:01	08:03	15:01
Patient 6	02:07	11:01	*13:01*	6:01	01:02	03:04	06:01	06:10	15:01	15:01
Patient 7	11:01	11:01	*13:01*	15:32	03:04	12:03	03:01	03:01	11:06	13:12
Patient 8	11:01	24:02	*13:01*	15:25	03:04	04:03	03:01	05:02	12:02	15:01

[1] Patient 3 PBMC were not available for HLA typing.

3.3.3 Medium for T-cell culture and cloning.

This medium has been described in detail in chapter 2 section 2.2.

3.3.4 Lymphocyte transformation test and PBMC ELIspot.

PBMC ($1.5x10^5$ cell/well) from hypersensitive patients and control donors were incubated with DDS (125-500 μM), DDS-NO (10-40 μM), rifampicin (10-100 μM), clofazimine (10-100 μM) or tetanus toxoid (5μg/mL, as a positive control) in culture medium for 5 days. [3H]-thymidine was added for the final 16h of the experiment. IFN-γ secreting PBMC were visualized using ELIspot (MabTech, Nacka Strand, Sweden) by culturing PBMC ($5x10^5$ cell/well) in medium with or without optimal concentrations of DDS or DDS-NO for 48 hrs.

3.3.5 Generation of EBV-transformed B-cells.

Epstein-Barr virus transformed B-cell lines were generated from PBMC and used as antigen presenting cells in experiments with T-cell clones (Wu et al., 2007). This method is described in chapter 2 section 2.3.3.

3.3.6 Generation of drug-specific T-cell clones.

Isolation of PBMC (as described in chapter 2 section 2.3.1) and generation of drug-specific T-cell lines and T-cell clones from PBMC which derived from DDS hypersensitive patients' blood samples are described in chapter 2 section 2.3.4. The cells were serially diluted (described in chapter 2 section 2.3.4.2). Initial proliferation tests were performed for picked clones using radioactive thymidine uptake assay.

3.3.7 Phenotype and specificity testing of drug-specific T-cell clones.

T-cell phenotyping was performed by flow cytometry (described briefly in chapter 2 section 2.3.11). TCR Vβ expression was measured using the IOTest® Beta Mark by flow cytometry with characterization of chemokines receptors for DDS- and DDS-NO-specific T-cells clones (chapter 2 section 2.3.11). Dose-dependent proliferative responses and the profile of secreted cytokines (chapter 2 section 2.3.5 and section 2.3.9).

DDS- and DDS-NO-responsive clones ($5x10^4$) were also cultured with non-toxic concentrations of structurally-related compounds (sulfamethoxazole, sulfamerazine, sulfadiazine, sulfachloropyridazine, sulfadoxin, sulphanilamide, 4, 4 thiodianiline, 4, 4 oxyaniline, 3, 3 sulfonyldianiline and mono and diacetylated forms of dapsone) and antigen presenting cells ($1x10^4$; 200 µL) for 48 hrs. Proliferation was measured by the addition of [3H] thymidine followed by scintillation counting.

3.3.8 Pathway of T-cell activation.

To explore the pathway of $CD4^+$ and $CD8^+$ T-cell activation with DDS and DDS-NO, T-cell clones were subjected to a variety of experimental protocols, mentioned in details in chapter 2. First, clones were cultured with optimal concentrations of DDS or DDS-NO in the presence or absence of antigen presenting cells (chapter 2 section 2.3.6). Second, MHC class I and class II restriction assay was performed (chapter 2 section 2.3.10). Third, clones were

cultured with DDS or DDS-NO and glutaraldehyde-fixed antigen presenting cells. Fixation blocks protein processing (chapter 2 section 2.3.7) (Zanni et al., 1998a). Fourth, antigen presenting cells pulsed with DDS or DDS-NO for 0.5-2 hrs were used to activate T-cells in the absence of soluble drug (chapter 2 section 2.3.8). Fifth, clones were cultured with DDS or DDS-NO and antigen presenting cells in the presence or absence of glutathione (1mM). Glutathione binds covalently to aromatic nitroso compounds, limiting their protein reactivity (Naisbitt et al., 1996b, Ellis et al., 1992). The stability of DDS and DDS-NO and the formation of dapsone glutathione adducts during the culture period were measured by mass spectrometry. Briefly, aliquots of cell culture supernatant (20 µL) and the calibration standards (20 µL) were diluted with LC-MS grade water (1:10 dilution) and deproteinized with acetonitrile. The extracts were evaporated to dryness in a Speed-Vac and reconstituted in 200 µL water. 5 µL samples and standards were analysed immediately by a Triple QuadTM 6500 mass spectrometer (AB Sciex,) coupled with a 1260 Infinity LC system (Agilent Technologies, Germany). The multiple reaction monitoring transitions for each analyte were as following: DDS 249.1/156.1 and 249.1/107.9; azoxy dapsone, 509.1/108.1 and 509.1/156.1; dapsone-GSH (Bergström et al., 2007), 602.1/401.3, 602.1/156.1, and 602.1/261.5. Other MS parameters, such as voltage potential and collision energy were optimised to achieve the greatest sensitivity. Data acquisition and quantification were performed using Analyst 1.5 software and Multi-Quant 3.0 (AB Sciex).

3.3.9 The involvement of HLA-B*13:01 in the activation of $CD8^+$ T-cell clones.

To explore whether DDS and DDS-NO interact with HLA-B*13:01 to activate $CD8^+$ T-cell clones, EBV-transformed B-cells were generated from 9 healthy donors expressing HLA alleles displaying at least 90% similarity to HLA-B*13:01 (Table 3.3). EBV-transformed B-cells from one additional hypersensitive patient expressing HLA-B*13:01 itself were also

used. $CD8^+$ T-cell clones were cultured with the different antigen presenting cells and DDS or DDS-NO for 48 hrs. Proliferation was measured by the addition of [^{3}H]-thymidine followed by scintillation counting.

Table 3-3 HLA typing of antigen presenting cells from healthy donors.

Alleles with ↑ 90% amino acid B*13:01 match	**Sequence similarity**	**HLA alleles expressed on antigen presenting cells from healthy donors**					
		HLA-A	**HLA-B**	**HLA-C**	**HLA-DRB1**	**HLA-DQB1**	**HLA-DQA1**
Donor 1 (B*08:01)	91.7	01:01 01:01	*08:01 57:01*	07:01 07:01	04:04 07:01	03:03 04:02	02:01 03:01
Donor 2 (B*13:02)	99.2	02:01 30:01	*13:02 57:01*	06:02 7:01	07:01 07:01	02:01 03:03	02:01 02:01
Donor 3 (B*15:01)	93.9	01:01 03:01	*15:01 57:01*	03:03 6:02	04:01 07:01	03:02 03:03	02:01 03:01
Donor 4 (B*35:01)	94.5	03:01 26:01	*35:01 35:01*	04:01 4:01	01:01 11:01	03:01 05:01	01:01 05:01
Donor 5 (B*40:01)	93.4	02:01 03:02	*40:01 51:01*	03:04 4:02	11:04 11:01	03:01 03:01	05:01 05:01
Donor 6 (B*44:02)	96.7	02:01 02:01	*44:02 57:01*	05:01 06:02	04:01 07:01	03:01 03:03	02:01 03:01
Donor 7 (B*46:01)	92.3	11:01 24:02	*46:01 58:01*	01:02 03:02	03:01 09:01	02:01 03:03	03:01 05:01
Donor 8 (B*51:01)	94.2	11:01 11:01	*51:01 52:04*	04:01 12:02	04:02 14:04	03:02 05:03	01:01 03:01
Donor 9 (B*58:01)	94.2	02:06 02:01	*15:01 58:01*	01:02 07:01	13:02 15:01	06:09 06:02	01:02 01:02
Donor 10[1] (B*13:01)	100		*13:01*				

[1] Antigen presenting cells from hypersensitive patients 5, 6 or 8 were used. See Table 3.2 for HLA typing data.

3.3.10 Statistics

All statistical analysis (One-way ANOVA unless stated otherwise) was performed using SigmaPlot 12 software (*$P<0.05$).

3.4 Results.

3.4.1 Patch testing of DDS hypersensitive patients.

Patch testing was conducted on the back of 6 patients with DDS concentrations between 0.1-25%. After 72 hrs drug exposure, 3 out of 6 patients displayed DDS concentration-dependent positive readings (Figure 3.1 A).

3.4.2 PBMC from DDS hypersensitive patients proliferate and secrete IFN-γ, following stimulation with DDS and DDS-NO.

PBMC from all 3 patch test positive patients were stimulated to proliferate strongly in the presence of DDS and DDS-NO (Figure 3.1B). Maximally tolerated concentrations of DDS and DDS-NO were 250-500μM and 40-50 μM, respectively. Positive DDS-specific proliferative responses (SI ≥ 2 or above) and/or IFN-γ secretion were also detected with PBMC from the 3 patch test negative patients, while DDS-NO responses were detected in 2 patients (Figures 3.1B-D). PBMC were not activated with the co-medications rifampicin and clofazimine (Figure 3.1D). Proliferative responses were also detected with the positive control phytohemagglutinin (results not shown).

PBMC from drug naïve HLA-B*13:01+ controls proliferated in the presence of phytohemagglutinin, but not the test drugs (SI less than 1.5; results not shown).

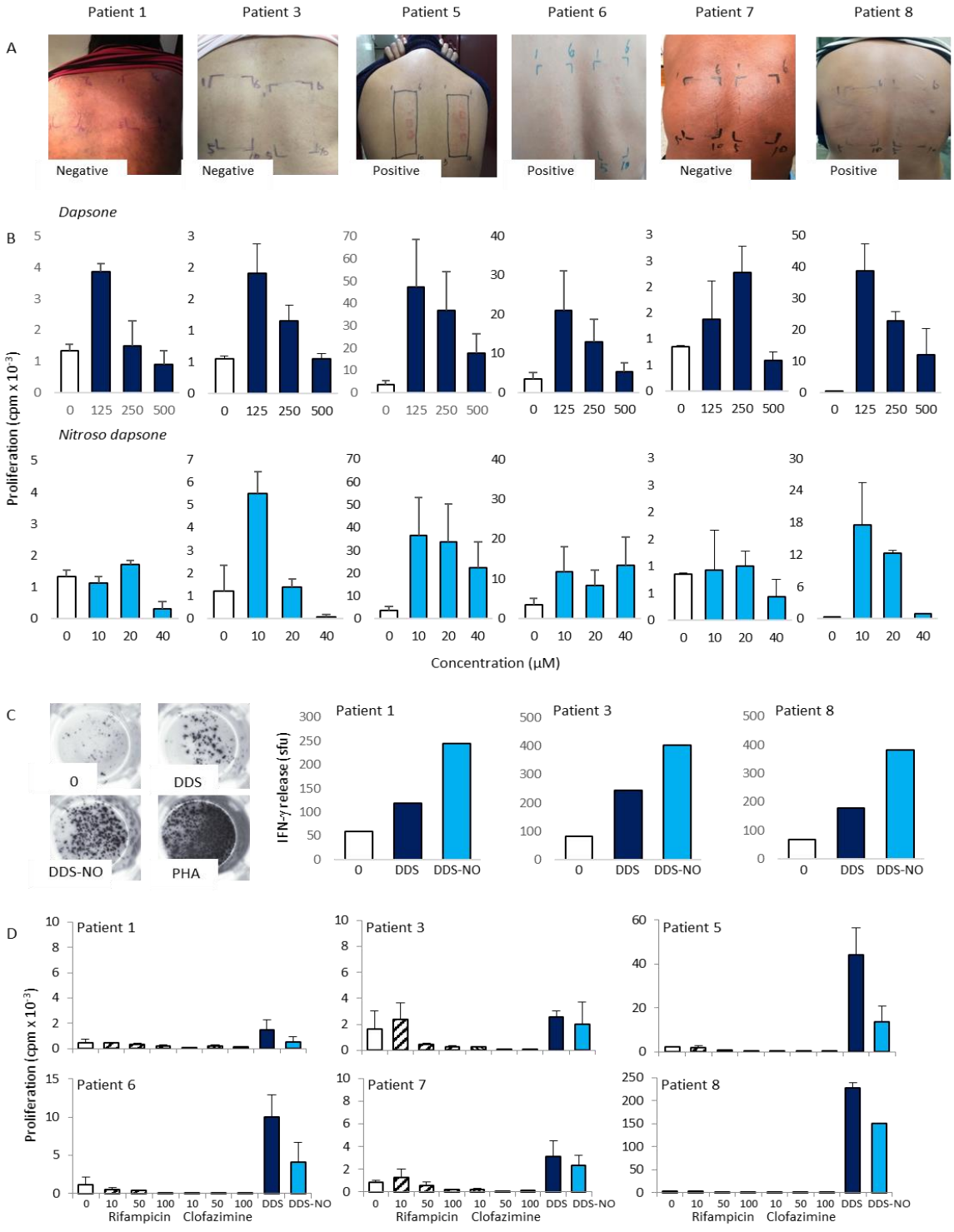


Figure 3-1 Diagnosis of DDS hypersensitivity by skin testing and in vitro assays. (A) Skin patch test results for DDS hypersensitivity. Skin was exposed to DDS in polyethylene glycol 200 at dilutions of 0, 0.1, 0.5%, 1, 5, 10, 15, 2%, and 25%. The patch tape was left to the skin for 48 hrs and the diagnosis was made 24 hrs later. (B and D) PBMC from patients were exposed to graded concentrations of DDS, DDS-NO, rifampicin and clofazimine. Proliferation was measured after 6 days by the addition of [^{3}H] thymidine. Results are expressed as mean±SD cpm of triplicate cultures. A doubling of cpm in drug-treated cultures over vehicle control is considered positive. (C) PBMC from patients were exposed to optimal concentrations of DDS or DDS-NO and IFN-γ release was visualized by ELIspot.

3.4.3 DDS and DDS-NO activate $CD4^+$ and $CD8^+$ T-cell clones.

A total of 1334 and 1374 $CD4^+$ and $CD8^+$ clones, respectively, were expanded from DDS and DDS-NO T-cell lines from patients 5, 6 and 8. Six hundred and twenty six $CD4^+$ clones displayed reactivity against DDS or DDS-NO (Figure 3.2). DDS- and DDS-NO-specific $CD8^+$ T-cell clones were generated in lower numbers; one hundred and sixty eight were activated with either the drug or drug metabolite. DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were detected in equal numbers (Table 3.4).

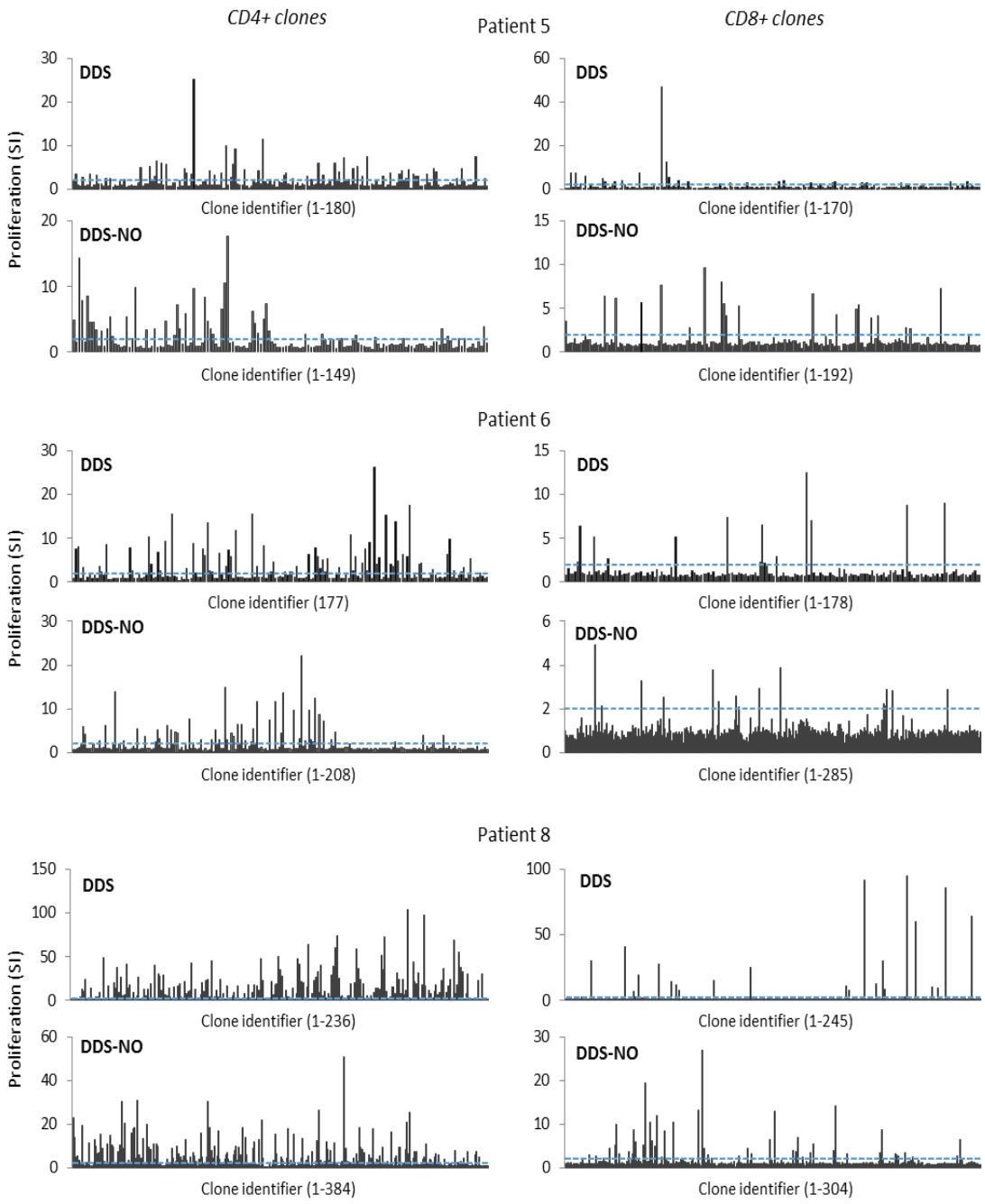


Figure 3-2 Generation of DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones. Clones were generated from DDS and DDS-NO CD4$^+$ or CD8$^+$ T-cell lines by serial dilution and repetitive mitogen stimulation. Well-growing clones were cultured with autologous antigen presenting cells and either DDS or DDS-NO in duplicate cultures for 48 hrs. Proliferative responses were measured by the addition of [^{3}H]-thymidine. Clones with a stimulation index (SI) of 2 or above were expanded further for phenotypic analysis and mechanistic studies.

Table 3-4 Phenotype and drug specificity of T-cell clones generated from dapsone hypersensitive patients.

Phenotype & drug specificity	**Total Number of clones**	**Number of drug-specific clones**	**Percentage of responding clones (%)**
Patient 8			
CD4+, DDS+	236	176	74.58
CD8+, DDS+	245	29	11.84
CD4+, DDS-NO+	384	210	54.69
CD8+, DDS-NO+	304	54	17.76
Patient 6			
CD4+, DDS+	177	76	42.94
CD8+, DDS+	178	14	7.87
CD4+, DDS-NO+	208	57	27.40
CD8+, DDS-NO+	285	15	5.26
Patient 5			
CD4+, DDS+	180	64	35.56
CD8+, DDS+	170	36	21.18
CD4+, DDS-NO+	149	43	28.86
CD8+, DDS-NO+	192	20	10.42

One hundred and two well-growing clones were selected for dose-response studies and the analysis of cytokine secretion. Proliferative responses were detected with 3 well tolerated concentrations of DDS (125-500 μM) and DDS-NO (5-20 μM) and drug treatment resulted in the secretion of Th1 (IFNγ), Th2 (IL-5, IL-13) and Th22 (IL-22) cytokines, alongside the cytolytic molecules perforin, granzyme B and FasL. $CD4^+$ and $CD8^+$ clones secreted similar levels of cytokines and cytolytic molecules (Figure 3.3). Table 3.5 shows the percentage of DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones that secrete individual cytokines.

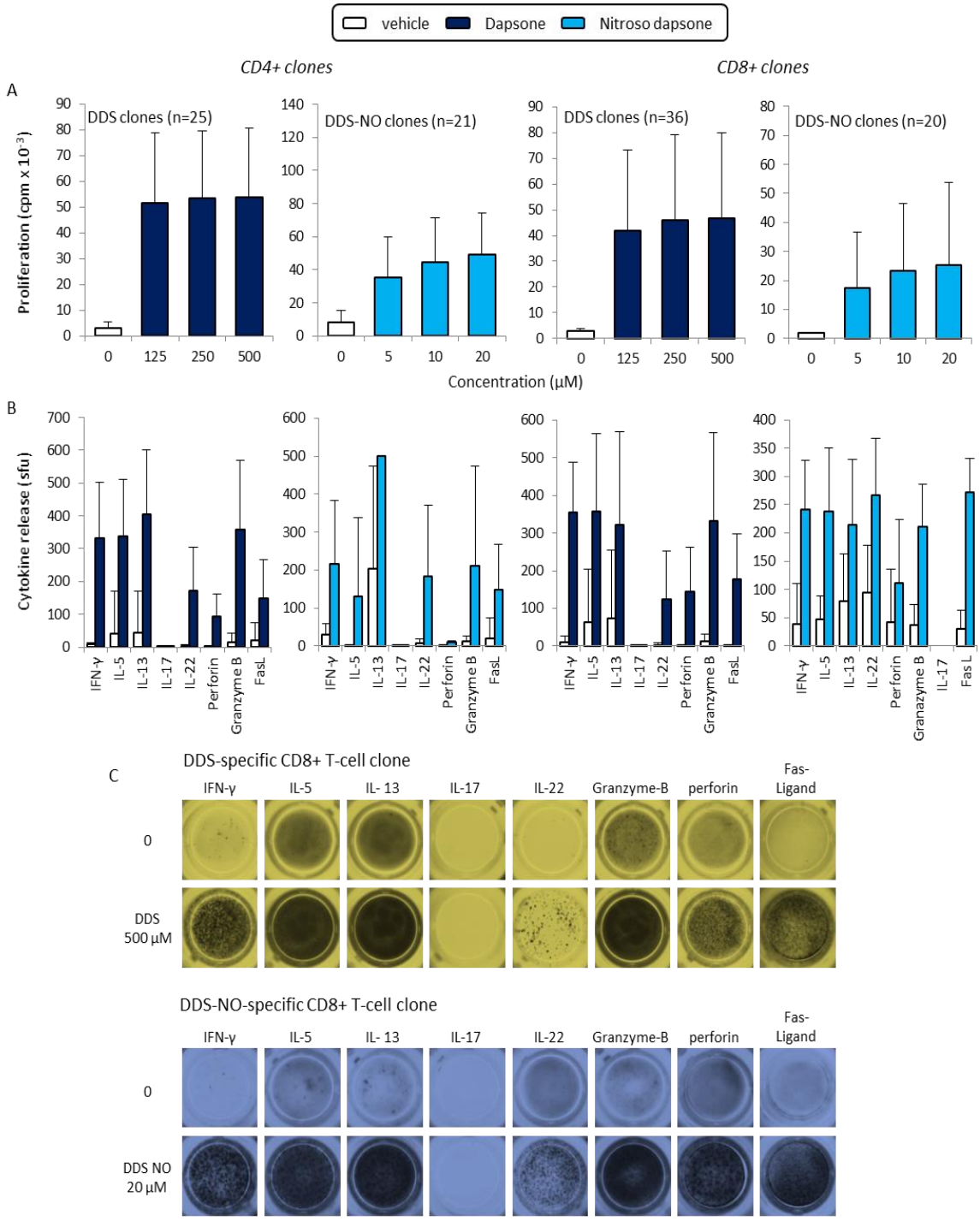


Figure 3-3 Dose-dependent proliferative response and cytokine release by DDS- and DDS-NO-responsive CD4⁺ and CD8⁺ T-cell clones. (A) One hundred and two CD4⁺ or CD8⁺ T-cell clones were incubated with autologous antigen presenting cells and either DDS (125-500 µM) or DDS-NO (5-20µM) in triplicate cultures for 48h. Proliferative responses were measured by the addition of [^{3}H] thymidine. Results are expressed as mean±SD cpm of the indicated number of clones. (B) Detection of IFN-γ, IL-5, IL-13, IL-17, IL-22, granzyme B, perforin and Fas-ligand secretion by DDS- and DDS-NO-responsive CD4⁺ and CD8⁺ T-cell clones. Clones were incubated with autologous antigen presenting cells and either DDS or DDS-NO and cytokine release was visualized by ELIspot. (C) Representative ELIspot images showing the cytokines released by DDS and DDS-NO-responsive CD8⁺ T-cell clones.

Table 3-5 Cytokine secretion from DDS and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones.

		IFN-γ	IL-5	IL-13	IL-17	IL-22	Per-forin	Granz-yme B	Fas L
CD4	**DDS**	100[1]	88	82	0	100	94	94	92
	DDS-NO	80	100	60	0	100	60	80	92
CD8	**DDS**	100	79	57	0	78	93	78	100
	DDS-NO	100	100	100	0	90	100	100	100

[1] Numbers refer to the percentage of clones with an increase of at least 50 sfu when the drug- and vehicle-treated wells were compared.

3.4.4 DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones display three distinct patterns of reactivity.

Sixty three DDS- and ninety eight- DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were assayed for crossreactivity. When all of the DDS-responsive clones were assessed together, low levels of proliferation was observed with DDS-NO (Figure 3.4 A). It should be noted that the maximum concentration of DDS-NO used was over 10 times lower than the DDS concentration. Analysis of individual clones revealed three distinct crossreactivity patterns; DDS-specific, and weakly and strongly crossreactive with DDS-NO. Approximately 90% of the clones were DDS-specific or weakly crossreactive (Figure 3.4 B and C).

DDS-NO $CD4^+$ and $CD8^+$ T-cell clones displayed a much higher level of crossreactivity (Figure 3.4 A). However, 3 patterns of crossreactivity were again observed with individual clones; DDS-NO-specific, and weakly and strongly crossreactive with DDS. In contrast to the DDS-responsive clones, approximately 90% of the DDS-NO-responsive clones were DDS-NO-specific or highly crossreactive (Figure 3.4 B and C).

DDS- and DDS-NO-responsive clones were also stimulated to proliferate with DDS hydroxylamine; however, proliferative responses were not detected when clones were cultured with (1) DDS analogues with substitutions in the sulfone group, (2) DDS analogues with amine groups in different positions on the aromatic rings and (3) structurally distinct sulfonamide antimicrobials (Figure 3.5).

The stability of DDS-NO in the 2 day proliferation assay was assessed. DDS-NO was converted rapidly to azoxy dimers and the parent compound. Both compounds were detectable within a 10 min incubation with antigen presenting cells and T-cell clones. After 2 days, 50% of DDS-NO had been converted to DDS (Figure 3.4 D-E).

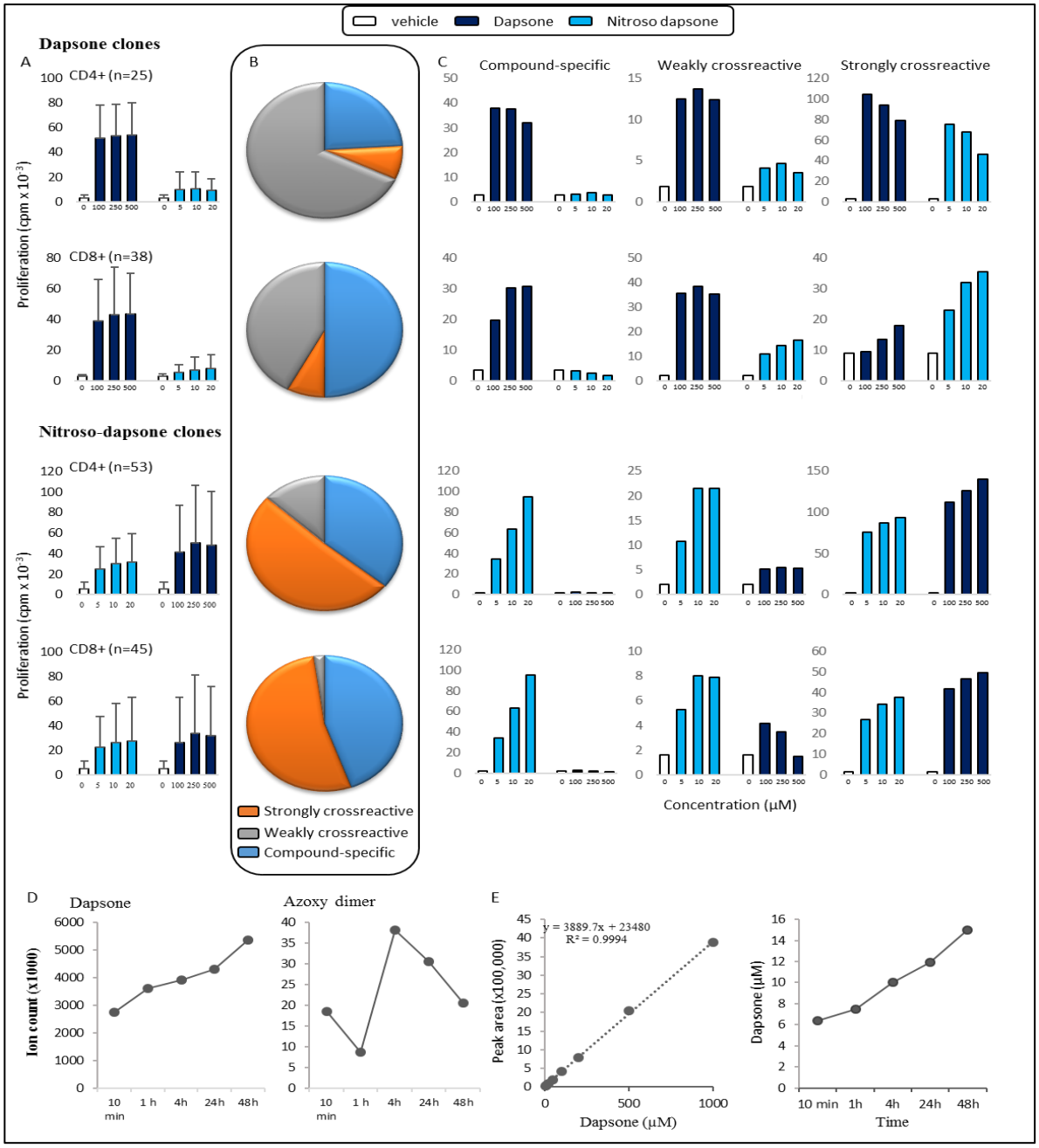


Figure 3-4 Crossreactivity of DDS- and DDS-NO-responsive T-cell clones. DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were cultured with irradiated autologous EBV-transformed B-cells and either DDS (100-500 µM) or the nitroso metabolite (5-20 µM) in triplicate cultures for 48h. T-cell proliferative responses were assessed through the addition of [^{3}H]-thymidine. (A) Mean crossreactivity data for 153 clones divided according to the drug antigen PBMC were cultured with drug to generate clones and CD phenotype. (B) Pie charts showing a number of clones with a particular crossreactivity profile (compound specific, weekly crossreactive (crossreactive compound displaying 10-50% response detected with a comparator) and strongly crossreactive (crossreactive compound displaying greater than 50% response detected with a comparator). (C) Representative clones displaying each response profile. (D) Relative quantification of DDS and azoxy dimer in cultures containing DDS-NO (30 µM), autologous EBV-transformed B-cells and T-cell clones. (E) Absolute quantification of DDS formation in cultures containing DDS-NO (30 µM), autologous EBV-transformed B-cells and T-cell clones. Left hand graph shows the standard curve for DDS (concentration range: 5nM-1µM). Right hand side graph shows the time-dependent formation of DDS.

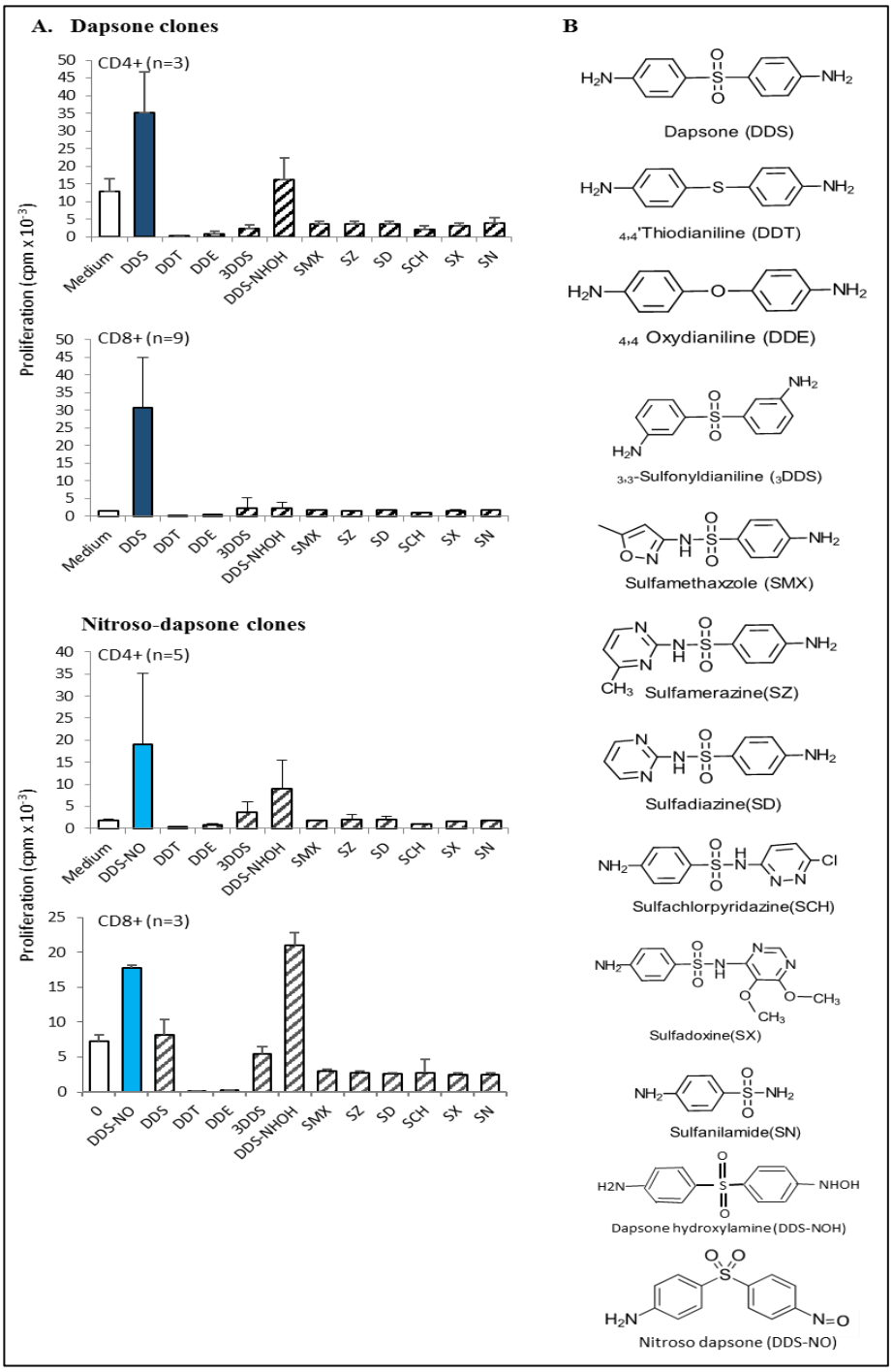


Figure 3-5 Crossreactivity of DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones with dapsone hydroxylamine, analogues and sulfonamide antimicrobials. (A) DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones were cultured with irradiated autologous EBV-transformed B-cells and non-toxic concentrations of the test compounds in triplicate cultures for 48 hrs. T-cell proliferative responses were assessed through the addition of [^{3}H]-thymidine. (B) Structure of the test compounds.

3.4.5 Crossreactive clones are activated with equivalent concentrations of DDS and DDS-NO.

Six strong and weakly crossreactive clones were incubated with DDS and DDS-NO at concentrations of 0.1-100 μM to define the minimum stimulatory concentrations of the two compounds. Surprisingly, clones were stimulated to proliferate with equivalent concentrations of each compound (Figure 3.6). The line graphs in Figure 3.6 A and B show the level of DDS and DDS-NO-specific proliferation for each clone on the same scale. The same data is then reproduced as bar charts on different scales to show more clearly that the clones are activated at the same concentrations of drug and metabolite irrespective of the extent of crossreactivity.

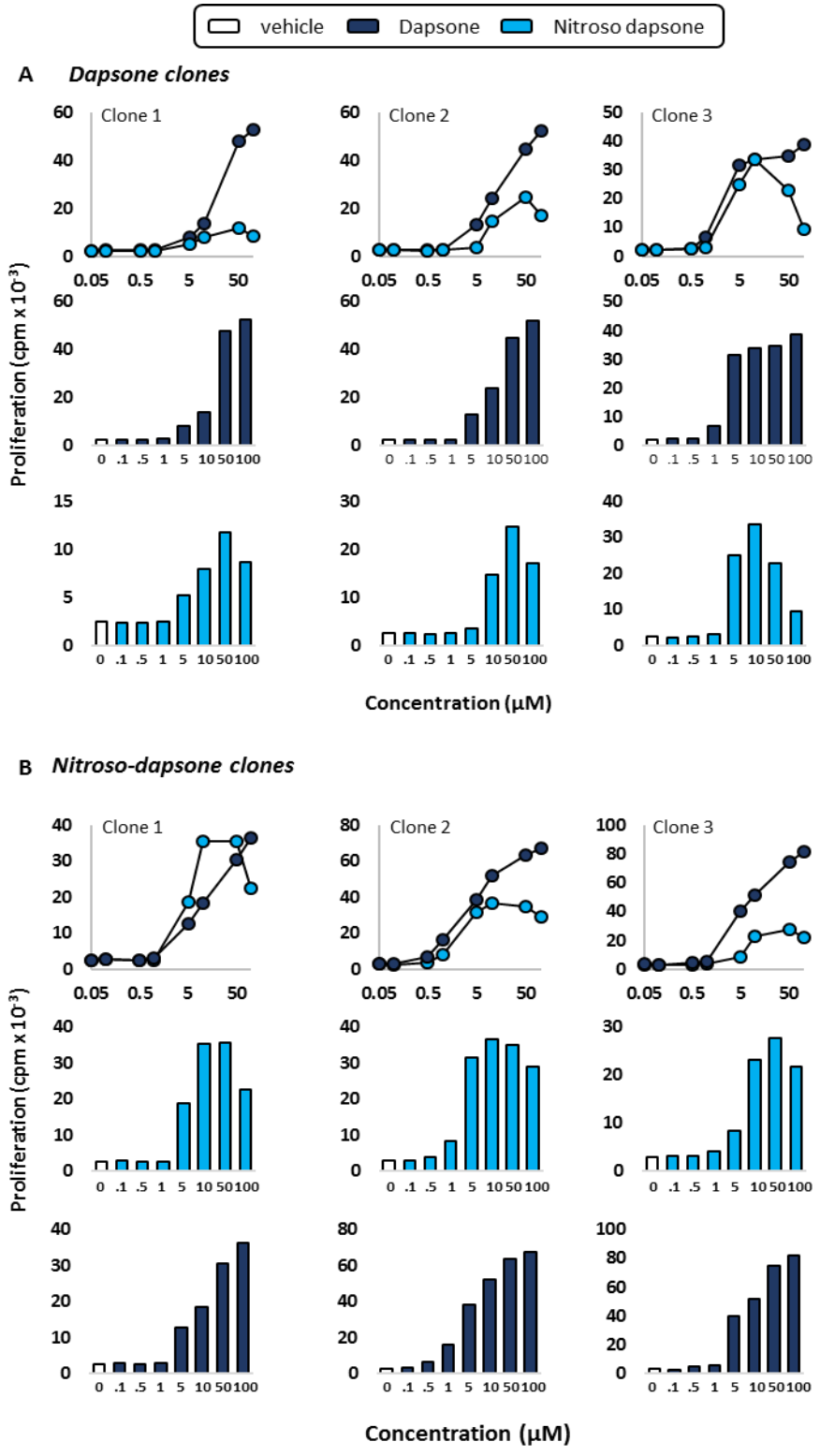


Figure 3-6 DDS and DDS-NO activate T-cell clones at the same minimum concentration. (A) DDS- and (B) DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were cultured with irradiated autologous EBV-transformed B-cells and either DDS (0.1-100 µM) or the nitroso metabolite (0.1-100 µM) in triplicate cultures for 48 hrs. T-cell proliferative responses were assessed through the addition of $[^3H]$-thymidine. Representative weakly and strongly cross-reactive DDS and DDS-NO-responsive clones are shown. Line graphs show proliferative responses to DDS and the nitroso metabolite on the same scale. Bar charts show the same data on different scales to compare minimum stimulatory concentrations.

3.4.6 DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones expressing multiple TCR Vβ chains display distinct chemokine receptor profiles.

DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones expressed single, but variable TCR Vβ chains (Figure 3.7 A), with no clear differences discernible when $CD4^+$ and $CD8^+$ or DDS- and DDS-NO-responsive clones were compared. DDS- and DDS-NO-responsive $CD4^+$ T-cell clones expressed high levels of the chemokine receptors CXCR3 and CCR4. The $CD8^+$ T-cell clones expressed CXCR3 and CCR4 alongside CCR10, CCR9 and CCR6 (Figure 3.7 B).

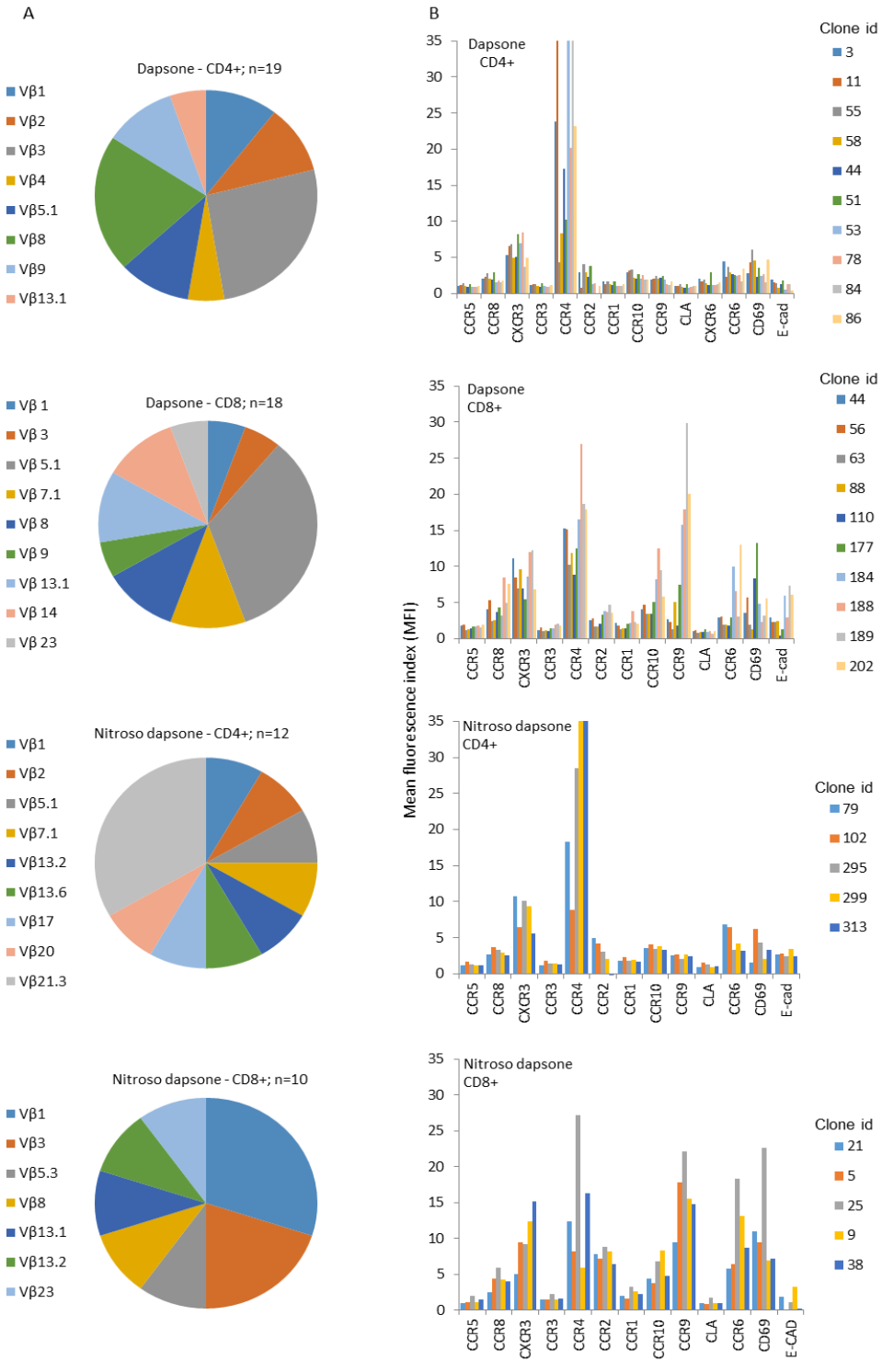


Figure 3-7 T-cell receptor Vβ chains and chemokine receptors expressed on DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones. (A) TCR Vβ expression was measured with the IOTest® Beta Mark, TCR Vβ Repertoire Kit, which covers over 80% of the available TCRs, by flow cytometry. (B) Chemokine receptors were measured on resting clones by flow cytometry. The expression is presented as mean fluorescence intensity of the whole population of each clone.

3.4.7 HLA-restricted activation of DDS- and DDS-NO-responsive clones ensues via different mechanisms.

Stimulation of DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were dependent on the presence of antigen presenting cells (i.e., irradiated autologous EBV-transformed B-cells; Figure 3.8 A). Use of anti-HLA class I and II blocking antibodies revealed that $CD4^+$ and $CD8^+$ proliferative responses to DDS and DDS-NO were HLA class II and I restricted, respectively (Figure 3.8 B). Fixation of antigen presenting cells with glutaraldehyde, which inhibits antigen processing, had no effect on the activation of $CD4^+$ or $CD8^+$ T-cell clones with DDS.

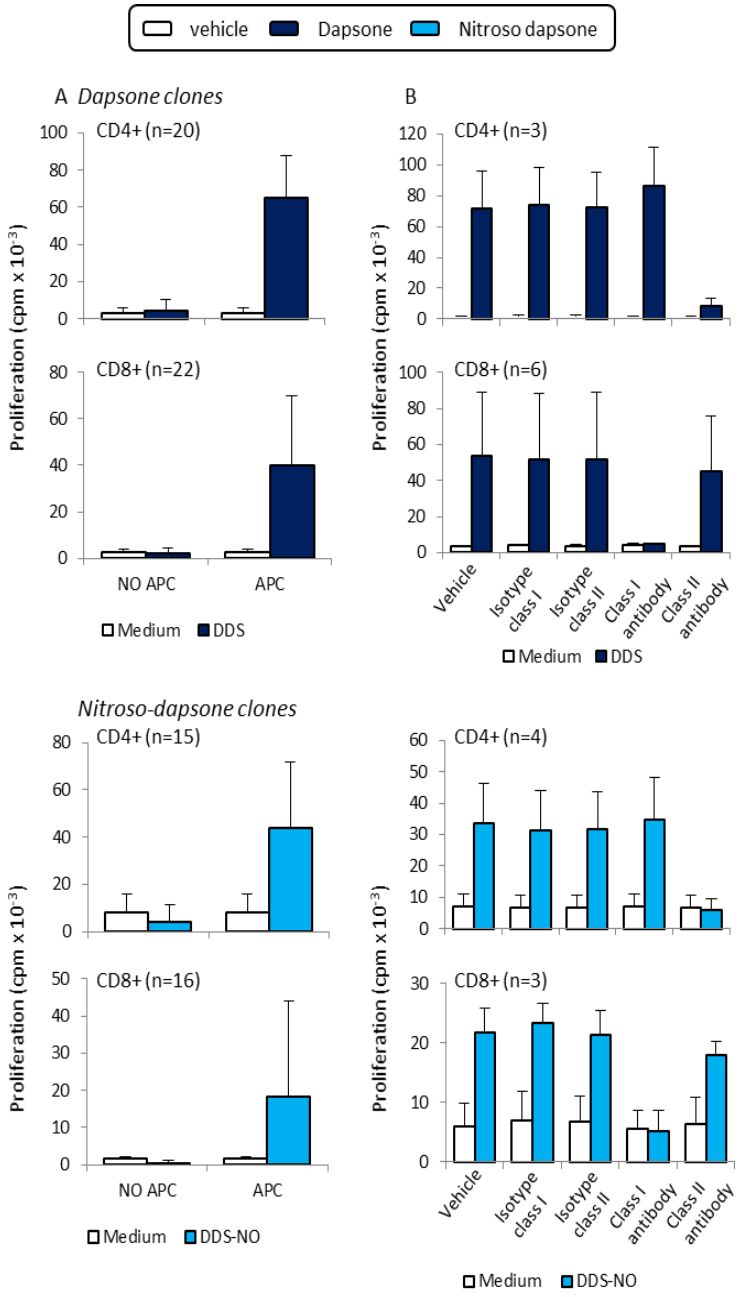


Figure 3-8 Antigen presenting cells are required for the activation of DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones. (A) DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones were cultured with DDS (500 µM) or the nitroso metabolite (20 µM) in triplicate cultures for 48h either in the presence or absence of antigen presenting cells. (B) DDS- and DDS-NO-responsive CD4$^+$ and CD8$^+$ T-cell clones were cultured with antigen presenting cells and DDS (500 µM) or the nitroso metabolite (20 µM) in triplicate cultures for 48 hrs either in the presence or absence of anti-HLA class I and II blocking antibodies. [^{3}H]-thymidine was added for 16hrs to measure drug-specific proliferative responses.

In contrast, antigen presenting cell fixation reduced the extent of proliferation with the nitroso metabolite (Figure 3.9 A). The residual response detected with DDS-NO and fixed antigen presenting cells likely relates to the conversion of DDS-NO to DDS in culture medium.

DDS-NO-responsive $CD8^+$ clones were activated with antigen presenting cells pulsed with DDS-NO for 0.5-2 hrs; clones were not exposed to the soluble drug metabolite in this experiment (Figure 3.9 B). Two out of four DDS-NO-responsive $CD4^+$ T-cell clones were also stimulated to proliferate with DDS-NO-pulsed antigen presenting cells. Antigen presenting cells pulsed with DDS for 0.5-2 hrs did not activate the DDS-responsive $CD4^+$ or $CD8^+$ T-cell clones.

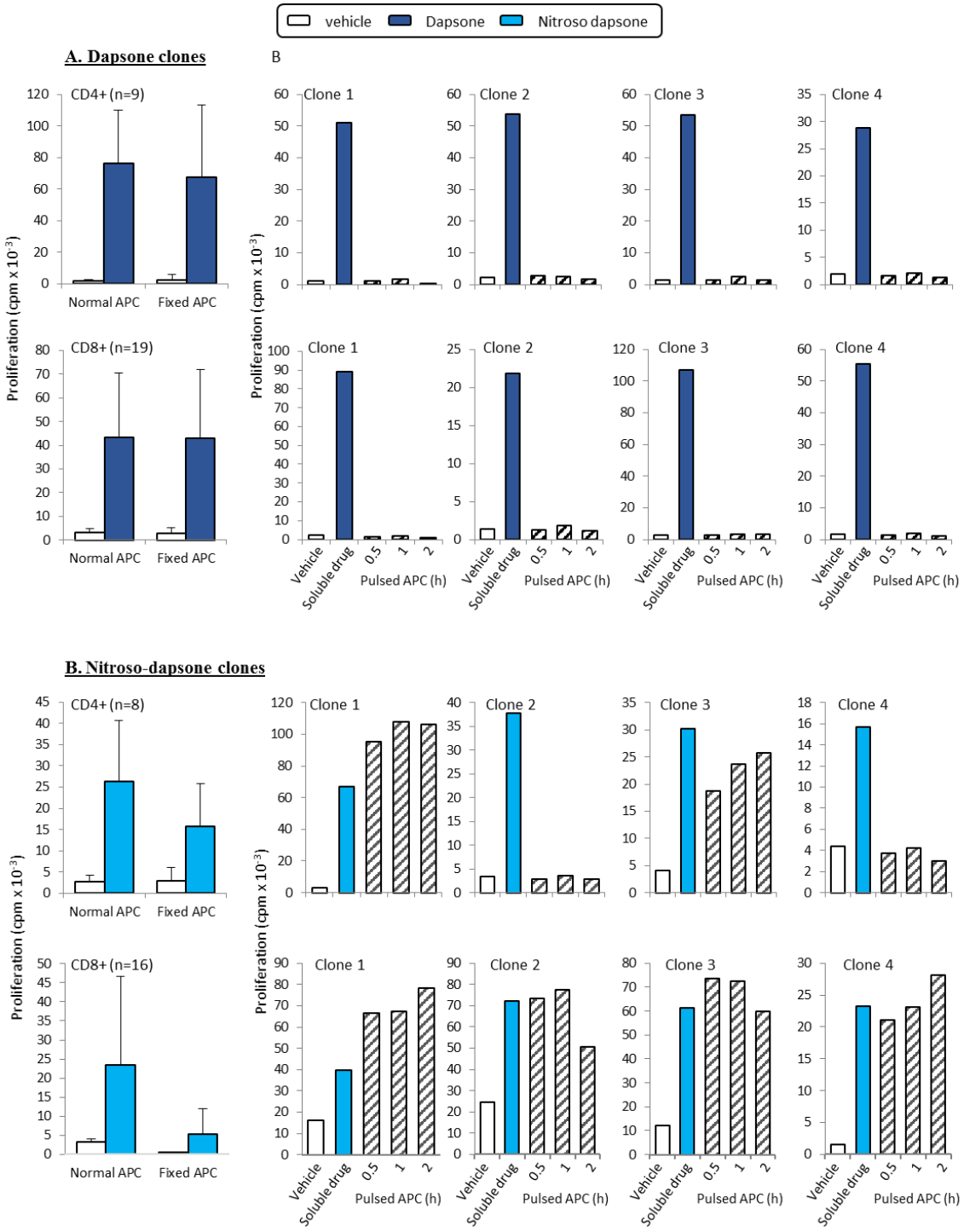


Figure 3-9 DDS and DDS-NO activate T-cells via different pathways. (A) DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were cultured with DDS or the nitroso metabolite in the presence of either irradiated or glutaraldehyde-fixed autologous EBV-transformed B-cells in triplicate cultures for 48 hrs. Fixation blocks antigen processing. (B) DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones were cultured with drug- or drug metabolite-pulsed (0.5-2 hrs) irradiated autologous EBV-transformed B-cells in the absence of soluble drug in triplicate cultures for 48hrs. T-cell proliferative responses were assessed through the addition of [^{3}H] thymidine.

Glutathione was added to (1) DDS-NO-responsive clones in a standard 48 hrs proliferation assay containing antigen presenting cells and soluble DDS-NO and (2) antigen presenting cells pulsed with DDS-NO for 2 hrs. Glutathione blocks the reactivity of aromatic nitroso compounds through reduction and conjugation reactions (Naisbitt et al., 1996b, Ellis et al., 1992). Figure 3.10 A compares the level of DDS-NO glutathione adducts formed naturally in the cell culture assay in the presence and absence of exogenous glutathione. Adducts were formed rapidly (within 2 hrs) and the level of adduct formation was several orders of magnitude higher in the presence of exogenous glutathione. Figure 3.11 contains traces showing how the adducts were quantified. The addition of glutathione to the T-cell assay containing soluble DDS-NO reduced the strength of the proliferative response, while the response to DDS-NO-pulsed antigen presenting cells was completely blocked (Figure 3.10 B).

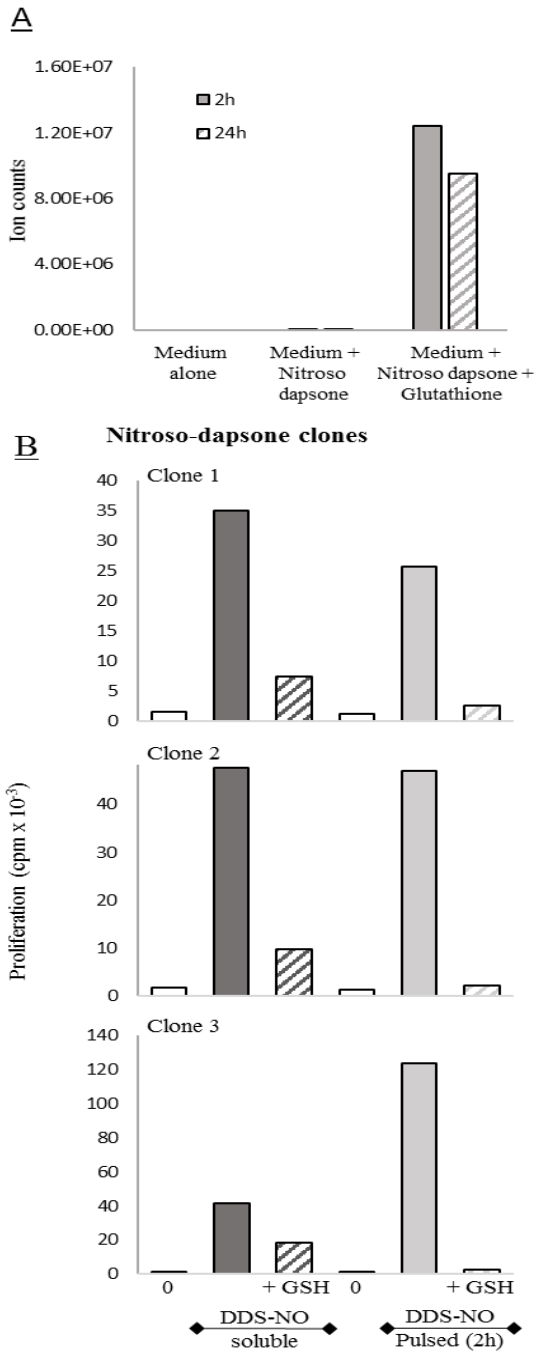


Figure 3-10 Glutathione inhibits the activation of T-cell clones with DDS-NO. (A) Semi-quantitative analysis of the levels of DDS-NO glutathione conjugate formed in incubations containing EBV-transformed B-cells in medium alone, medium containing DDS-NO or DDS-NO and glutathione (1mM). Figure 3.11 contains the mass spectrometric images. (B) DDS-NO-responsive clones were cultured with (i) irradiated autologous EBV-transformed B-cells, soluble drug metabolite and glutathione, and (ii) drug metabolite-pulsed (2 hrs) irradiated autologous EBV-transformed B-cells (±glutathione) in triplicate cultures for 48hrs. Glutathione was added to cells before DDS-NO in both assays. T-cell proliferative responses were assessed through the addition of [^{3}H]-thymidine.

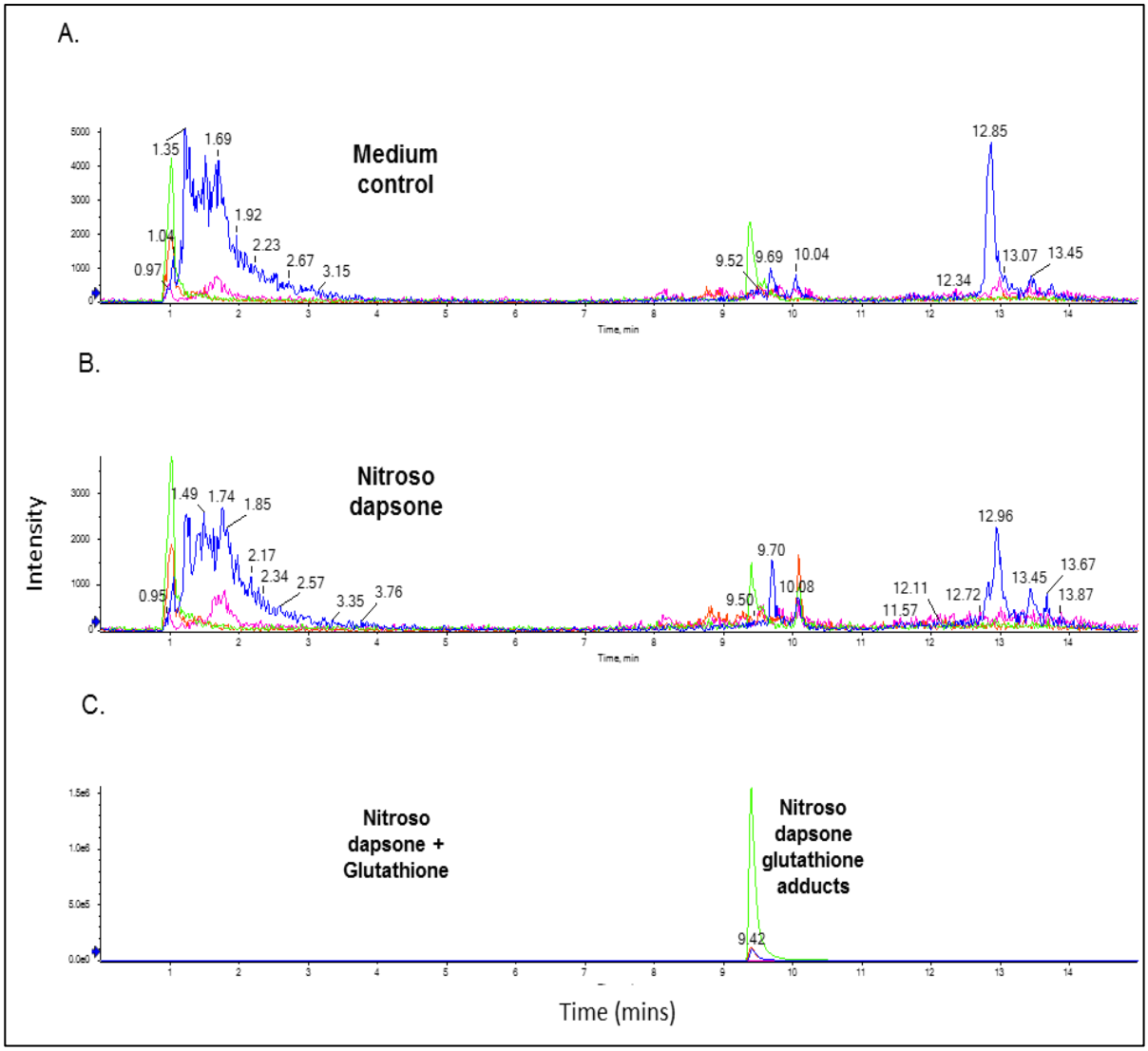


Figure 3-11 Mass spectrometric analysis of the DDS-NO glutathione adduct formed in the culture medium. EBV-transformed B cells were cultured with either (A) medium only, (B) medium containing DDS-NO or (C) DDS-NO in the presence of glutathione (1mM).

3.4.8 DDS and DDS-NO bind with a degree of selectively to HLA-B*13:01-dependent activation of CD8$^+$ T-cell clones.

EBV-transformed B-cells were generated from 9 healthy donors expressing HLA alleles with greater than 90% sequence homology to HLA-B*13:01. HLA typing of the healthy donors is shown in Table 3.3. The B-cell lines were used as antigen presenting cells in proliferation assays with DDS- and DDS-NO-responsive CD8$^+$ T-cell clones to explore the requirement

for HLA-B*13:01 for T-cell activation. Autologous antigen presenting cells and antigen presenting from cells 1 additional patient (both expressing HLA-B*13:01) were used as comparators.

As has been described previously for other drugs (von Greyerz et al., 2001a, Zanni et al., 1998a) 30% of DDS and DDS-NO-responsive HLA-class I restricted $CD8^+$ T-cell clones displayed proliferative responses with the drug or metabolite and antigen presenting cells expressing a wide variety of HLA alleles (Figure 3.12).

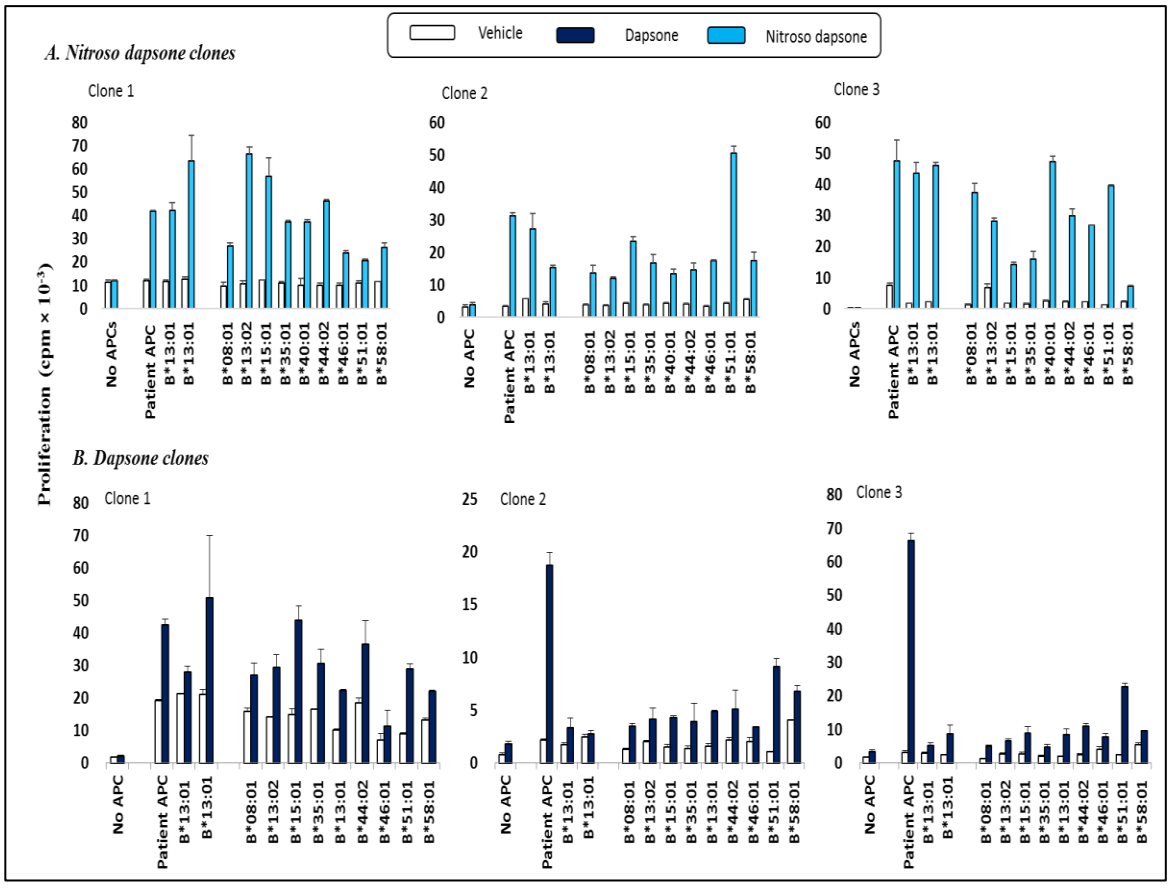


Figure 3-12 DDS and DDS-NO bind to wide variety of HLA alleles to activate certain $CD8^+$ T-cell clones. (A) DDS-NO- and (B) DDS-responsive $CD8^+$ clones were cultured with drug or drug metabolite and irradiated EBV-transformed B-cells from 12 donors expressing different HLA-B alleles in triplicate cultures for 48hrs. The complete HLA type of the different donors is shown in Table 3.3. $CD8^+$ T-cell clones were cultured with DDS or DDS-NO and irradiated EBV-transformed B-cells from 12 individuals expressing various HLA-B allele in triplicate cultures for 48hrs. T-cell proliferative responses were assessed through the addition of [^{3}H] thymidine.

Other clones were activated with the drug or metabolite only in the presence of autologous antigen presenting cells. Despite this, 30-40% of $CD8^+$ clones displayed a degree of HLA-B*13:01 allele restriction. Figure 3.13 A and B shows 3 clones (1 DDS- and 2 DDS-NO-responsive) that were stimulated to proliferate exclusively in the presence of DDS or DDS-NO and antigen presenting cells expressing HLA-B*13:01. Three additional clones (1 DDS- and 2 DDS-NO-responsive) are shown that were activated in the presence of antigen presenting cells expressing HLA-B*13:01 and either B*13:02, B*58:01 or B*51:01.

The 2 DDS responsive $CD8^+$ T-cell clones were expanded in sufficient numbers for us to conduct a proliferation assay using antigen presenting cells from the six hypersensitive patients that all express HLA-B*13:01. The clones were activated in the presence of DDS and antigen presenting cells from all of the patients (Figure 3.13 C).

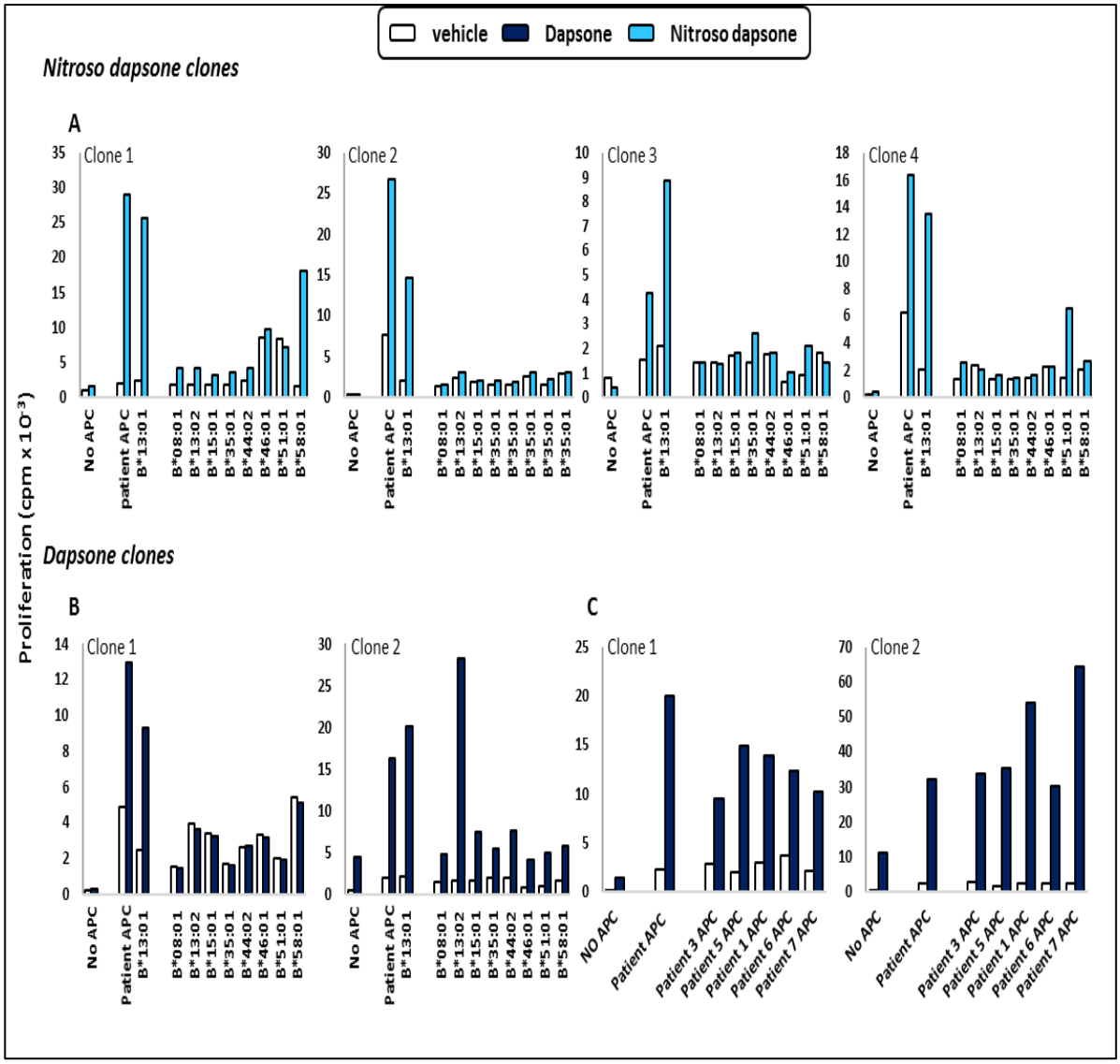


Figure 3-13 DDS and DDS-NO bind with a degree of selectively to HLA-B*13:01 to activate certain CD8$^+$ T-cell clones. (A) DDS-NO- and (B) DDS-responsive CD8$^+$ T-cell clones were cultured with drug or drug metabolite and irradiated EBV-transformed B-cells from 10 donors expressing different HLA-B alleles in triplicate cultures for 48 hrs. The complete HLA type of the different donors is shown in Table 3.3. (C) DDS-responsive CD8$^+$ T-cell clones were cultured with DDS and irradiated EBV-transformed B-cells from 6 hypersensitive patients expressing HLA-B*13:01 in triplicate cultures for 48 hrs. T-cell proliferative responses were assessed through the addition of [^{3}H]-thymidine.

3.5 Discussion.

Knowledge of the role drug metabolism plays in the generation of antigenic determinants that activate T-cells is limited. Circumstantial evidence supporting a role for drug metabolism includes (1) the identification of protein-reactive metabolites for many drugs associated with a high incidence of hypersensitivity and (2) the induction of toxicity in target tissue by reactive metabolites and hence the potential to disrupt immune regulatory pathways through the provision of danger signals (Uetrecht and Naisbitt, 2013). However, the most direct evidence linking drug metabolism to the development of hypersensitivity is the detection of halothane-specific antibodies in patients with liver failure (Kenna et al., 1993) and the observation that halothane protein adducts activate T-cells (You et al., 2010). Furthermore, halothane derivatives that form lower levels of reactive metabolite are associated with a decreased risk of liver injury (Park et al., 1998). To explore whether metabolites activate hypersensitive patient T-cells in vitro, co-culture strategies have been developed using microsomal metabolite generating systems (Bergström et al., 2007, Sachs et al., 2001). Enhanced drug-specific T-cell responses have been reported with drugs in the presence of a metabolising system; however, it is very difficult to delineate the disparate effects of the parent drug and metabolites. For this reason, we synthesized the reactive metabolite of sulfamethoxazole (Naisbitt et al., 1996a) which causes immune-mediated skin and liver reactions that are not linked to the expression of a specific HLA allele (Alfirevic et al., 2009). Working alongside other researchers in the field we have shown that PBMC and inflamed tissue from all hypersensitive patients contain T-cells that are activated with sulfamethoxazole and/or its reactive metabolite via different pathways (i.e., direct HLA binding and a hapten pathway, respectively) (Nassif et al., 2004a, Castrejon et al., 2010a, Schnyder et al., 2000).

In recent years, researchers studying hypersensitivity reactions strongly linked to the expression of specific HLA alleles have focussed exclusively on the interaction between parent drug and the HLA molecule. This is because the parent drug is, for the most part, the only reagent available for in vitro cell culture studies. The observation that T-cells from patients with hypersensitivity to drugs such as carbamazepine are activated with the parent drug (Ko et al., 2011), has lead researchers to hypothesize that reactive metabolites are not involved in the development of HLA allele-restricted forms of drug hypersensitivity. To investigate this hypothesis we now focused on DDS hypersensitivity in patients with leprosy. DDS is a model study drug as (1) hypersensitivity reactions in patients are strongly linked to the expression of HLA-B*13:01 (Zhang et al., 2013), (2) the reactive nitroso metabolite of dapsone has been synthesized in a stable form (Alzahrani et al., 2017b) and (3) patient samples are available for mechanistic studies. Utilizing PBMC from HLA-B*13:01+ patients we show $CD4^+$ and $CD8^+$ T-cell responses to dapsone and the nitroso metabolite. Furthermore, DDS and DDS-NO bind to HLA-B*13:01 to selectively activate certain $CD8^+$ T-cell clones.

Six HLA-B*13:01+ patients that developed hypersensitivity whilst receiving DDS in combination with rifampicin and clofazimine were recruited to the study. Three displayed a positive patch test response to DDS. PBMC from all six patients were stimulated to proliferate and/or secrete IFN-γ after stimulation with DDS or the nitroso metabolite. The strongest in vitro responses were seen in the patients with the positive patch test response and these patients were selected for serial dilution experiments to search for drug- and drug metabolite-responsive T-cell clones. $CD4^+$ and $CD8^+$ clones were purified prior to plating single cells in culture plates for the cloning procedure. DDS- and DDS-NO-responsive T-cells were generated in almost equal numbers (DDS 395; DDS-NO 399). The ratio of DDS- or DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones was 4-5:1 in patients 6 and 8 and 2:1

in patient 5, which demonstrates that drug exposure results in a complete cellular immune response against the drug antigens. This is in stark contrast to the findings with abacavir where $CD8^+$ T-cells are the only cells activated with the drug (Bell et al., 2013, Lucas et al., 2015). The activation of $CD4^+$ and $CD8^+$ T-cell clones that expressed an array of TCR Vβ receptors, with DDS or DDS-NO, was HLA class II and class I restricted, respectively. This confirms our previous data with PBMC from healthy donors (Alzahrani et al., 2017b), which shows that both DDS and DDS-NO interact with HLA class I and class II molecules.

The availability of cloned $CD4^+$ and $CD8^+$ T-cells permitted the detailed analysis of crossreactivity between DDS, DDS-NO and structurally-related compounds. The position of the amine groups on the aromatic rings and the availability of the sulfone group was found to be critical for the activation of the T-cell clones. A panel of sulfonamides, which contain one aromatic amine group did not activate the clones. Thus, both of dapsones' aromatic amines and sulfone were required for T-cell activation.

25-50% of $CD4^+$ and $CD8^+$ T-cell clones were classified as highly specific as they were only activated with one compound (either the parent drug or metabolite [Figure 3.4 B]). This confirms that T-cells recognize and respond selectively to the two different forms of the DDS antigen. DDS has an unusual structure in that the two aromatic amines connected to the sulfone group are identical. If the structure of a DDS-NO-modified HLA binding peptide is compared with DDS complexed to the same peptide, it is likely that similar conformations will be observed and as such the interaction with the T-cell receptor will be the same. Thus, our working hypothesis to explain the drug- or drug-metabolite-specific activation of certain clones is that the HLA binding peptides also participate in the TCR interaction and impart a degree of selectivity.

When using optimum concentrations of DDS (100-500 μM) and DDS-NO (5-20 μM) to activate $CD4^+$ and $CD8^+$ T-cells, clones displaying high (i.e., at least 50% of the response detected with the opposite compound) and low (i.e., 10-50% of the response of the response detected with the opposite compound) levels of crossreactivity were also detected. The majority of DDS-responsive, crossreactive $CD4^+$ and $CD8^+$ T-cell clones displayed low levels of crossreactivity with DDS-NO. Using quantitative mass spectrometry we were able to demonstrate that the nitroso metabolite is reduced to DDS in the 2 day proliferation assay. Thus, clones incubated with 30 μM DDS-NO were exposed to 2.5-15 μM of the parent drug, which stimulates a sub-optimal T-cell proliferative response. In contrast, the majority of crossreactive DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cell clones displayed high levels of crossreactivity with 100-500 μM DDS. To investigate the response profiles further, a panel of crossreactive DDS- and DDS-NO-responsive clones were cultured with equal concentrations of the parent drug and metabolite (0.1-100 μM). Interestingly, weakly and strongly crossreactive clones were activated with the same concentrations of DDS and DDS-NO with similar dose-response curves until nitroso metabolite-induced toxicity was detected. These data are in contrast to our earlier studies with sulfamethoxazole and its nitroso metabolite, where the parent drug activates T-cells at significantly higher concentrations (Castrejon et al., 2010a). It is reasonable to assume that HLA binding peptides play a less important role in the triggering of crossreactive TCR.

Antigen presenting cells were required for the strong activation of $CD4^+$ and $CD8^+$ T-cell clones with DDS and the nitroso metabolite. Clones were activated with DDS in the presence of irradiated and glutaraldehyde-fixed antigen presenting cells. In contrast, fixation reduced the strength of the DDS-NO-specific T-cell proliferative response. As discussed above, the best explanation for the weak response of DDS-NO-responsive clones with soluble DDS-NO and fixed antigen presenting cells is the formation of DDS in the proliferation assay.

Application of drug- or drug metabolite-pulsed antigen presenting cells instead of the soluble drug in T-cell assays provides a tool to differentiate between the effects of covalent and non-covalent forms of drug (Schnyder et al., 2000, Castrejon et al., 2010b). DDS-responsive clones were not activated with DDS-pulsed antigen presenting cells. In contrast, six out of the eight DDS-NO-responsive clones tested were activated with DDS-NO-pulsed antigen presenting cells and the strength of the induced response was the same as that seen with the parent drug. Two of the DDS-NO-responsive clones were not activated with pulsed antigen presenting cells; interestingly, both of these clones crossreacted strongly with DDS.

Glutathione is a tripeptide intracellular antioxidant that protects cells from exposure to aromatic nitroso compounds by acting as a reducing agent and through direct conjugation (Naisbitt et al., 1996a, Cribb et al., 1991, Ellis et al., 1992). The addition of glutathione to T-cell assays prevents the covalent binding of nitroso compounds and hence it is possible to explore whether T-cells are activated with drug metabolite-modified protein adducts (Burkhart et al., 2001). DDS-NO glutathione adducts were formed rapidly in cultures containing the drug metabolite, T-cells, antigen presenting cells and glutathione. Moreover, glutathione decreased the response of clones to soluble DDS-NO and blocked the response to DDS-NO-pulsed antigen presenting cells.

In the next section of the project, antigen presenting cell were generated from healthy donors expressing HLA-B alleles with at least 90% sequence homology to HLA-B*13:01 (including an additional HLA-B*13:01+ donor) to investigate whether either DDS or DDS-NO interact with a degree of selectivity to the risk allele to activate $CD8^+$ T cell clones. As described previously with other forms of drug hypersensitivity, several clones displayed HLA allele-unrestricted drug recognition (i.e., DDS and DDS-NO stimulated a proliferative response in the presence of multiple antigens presenting cells expressing different B alleles) (von Greyerz et al., 2001a). Other clones were only activated in the presence of DDS-NO and

autologous antigen presenting cells. However, a panel of clones were selectively activated with DDS or DDS-NO in the presence of antigen presenting cells displaying HLA-B*13:01. This indicates that both the parent drug and reactive metabolite interacts with HLA-B*13:01 to activate certain $CD8^+$ T-cell clones. We are therefore synthesizing designer DDS-NO-modified HLA-B*13:01 binding peptides and conducting structural analyses to compare the binding interaction of DDS and DDS-NO with HLA-B*13:01.

$CD4^+$ and $CD8^+$ T-cell clones secreted the same panel of cytokines when stimulated with DDS or DDS-NO, but expressed distinct chemokine receptors. Clones secreted Th1, Th2 and Th22 cytokines alongside the cytolytic molecules perforin, granzyme B and FasL; however, IL-17 was not detected. IL-22 secretion in the absence of IL-17 seems to be a common feature of drug-specific clones as this profile has now been detected with DDS, sulfamethoxazole and piperacillin (Sullivan et al., 2018, Gibson et al., 2014). The chemokine receptors displayed on DDS- or DDS-NO-responsive $CD4^+$ T-cell clones was restricted to CXCR3 and CCR4. In contrast, $CD8^+$ T-cell clones also expressed CCR6, 9 and 10. CCR4 and CCR10 have been implicated in the migration of T-cells into skin (Xia et al., 2014, Zaid et al., 2017); thus, a more detailed investigation of migratory properties of $CD4^+$ and $CD8^+$ T-cells in patients with DDS hypersensitivity is warranted.

In conclusion, our study shows that DDS- and DDS-NO-responsive $CD4^+$ and $CD8^+$ T-cells circulate in hypersensitive patients. T-cells were activated selectively with the parent drug and drug metabolite via direct HLA binding and a hapten mechanism, respectively. The detection of HLA-B*13:01-restricted DDS- and DDS-NO-responsive $CD8^+$ T-cell clones indicates that DDS hypersensitivity should be used as an exemplar to explore the structural features of drug HLA binding and how this interaction results in an aberrant T-cell response.

Chapter 4: Investigation of the immunological mechanisms relating to tolvaptan-induced liver injury.

4.1 Introduction.

Adverse drug reactions represent an important clinical problem and a major impediment to the drug development process (Park et al., 2011a). Drug-induced liver (DILI) is of major concern both in the clinic and during drug development. Immunologically-mediated reactions are one of the most feared as they are difficult to predict and they show no simple dose-response relationship. We have previously undertaken a number of studies to demonstrate the presence of drug-responsive T-lymphocytes in blood and skin of patients with cutaneous reactions, thus providing direct evidence for their involvement in the disease pathogenesis (Castrejon et al., 2010a, El-Ghaiesh et al., 2011, Elsheikh et al., 2011, Nassif et al., 2004b, Pichler, 2005, Schnyder et al., 2000, Schnyder et al., 1997, Whitaker et al., 2011, Wu et al., 2007, Wu et al., 2006). The role of T-cells in reactions targeting the liver is less well defined partly because of the lack of appropriate studies. In 1997, Maria and Victorino (Maria and Victorino, 1997) described lymphocyte responses to drugs in over 50% of patients with a drug-induced liver injury. More recently, histological examination of inflamed liver from a patient exposed to sulfasalazine revealed an infiltration of granzyme B secreting T-lymphocytes (Mennicke et al., 2009). In recent years, utilizing the experimental approach detailed in this proposal, we have detected and fully characterized drug-specific T-cells in patients with flucloxacillin, co-amoxiclav, isoniazid, ethanbutol and ticlopidine-induced liver injury (Usui et al., 2017b, Usui et al., 2018, Usui et al., 2016, Monshi, 2013).

It is thought that to activate immune cells, a drug must bind to HLA molecules and in some way crosslink specific T-cell receptors. The hapten hypothesis, based on the studies of Landsteiner and Jacobs relating sensitization potential to protein reactivity states that a chemical (drug) must bind covalently to self-protein to break immune tolerance (Landsteiner and Jacobs, 1935). It is suggested that T-cells are subsequently stimulated by peptides

liberated from the modified protein following antigen processing. Drugs have also been shown to associate directly with HLA and/or HLA binding peptides to stimulate a T-cell response (Elsheikh et al., 2010, Naisbitt et al., 2003). An improved understanding of drug-induced liver injury requires an integrated approach that relates drug disposition, drug antigenicity, drug immunogenicity, genetics and immune responses to clinical outcome. The recently described genetic associations between expression of particular HLA alleles and susceptibility to DILI (Chung et al., 2004b, Daly et al., 2009, Mallal et al., 2002) indicate that functional studies should be conducted with samples from HLA-typed individuals.

For immunological drug induce liver injury disease status and host factors represent risk factors; however, such factors are often poorly predictive of toxicity. Recently, several strong associations between susceptibility to DILI and expression of HLA alleles have been identified (e.g., flucloxacillin (Daly et al., 2009), ximelagatran (Kindmark et al., 2008), lumiracoxib (Singer et al., 2010), and lapatinib (Spraggs et al., 2011), which imply a direct effect of the gene product on the disease pathogenesis. These findings suggest that a major susceptibility factor relates to the restriction of the fit of the causative antigen into particular immunological receptors in an appropriate chemical form. For certain drugs that cause cutaneous reactions (i.e., abacavir, carbamazepine and DDS in this thesis), it has been possible to relate the genetic association to mechanisms of disease by characterizing drug-specific T-cell responses in volunteers carrying the appropriate HLA allele (Chessman et al., 2008, Ko et al., 2011). In contrast, HLA-restricted T-cell responses in patients with DILI are less well described. We have recently shown that flucloxacillin preferentially activates cytotoxic $CD8^+$ T-cells from patients with liver injury in an HLA risk allele (i.e., HLA-B*57:01) restricted fashion (Kim et al., 2015, Monshi Manal et al., 2012). Using cells from healthy donors, it is also possible to prime naïve $CD8^+$ T-cells to flucloxacillin with autologous dendritic cells expressing HLA-B*57:01 (Monshi et al., 2013b). In contrast to

the DILI patient cells that are activated via a hapten mechanism, T-cells from healthy donors can be activated via a direct binding interaction with HLA-B*57:01 (Faulkner et al., 2012b).

Tolvaptan (TVP) an orally effective nonpeptide arginine vasopressin (AVP) V2-receptor antagonist is an example of a drug that causes serious liver injury in certain rare patients. The delayed nature of the liver injury that has been observed with TVP, the prompt recurrence of injury, which is sometimes observed on rechallenge with tolvaptan, and the very preliminary HLA associations suggested by genetic analyses already undertaken, support the idea that the final and critical event underlying the injury is an adaptive immune attack on the liver. Thus, a hypothesis for TVP-induced DILI is that the drug or a derived metabolic product(s) forms antigenic determinants with MHC molecules to activate T-cells.

4.2 Aim of the study.

- The purpose of this work is to detect, isolate, and characterize T-cells that are functionally responsive to tolvaptan-derived antigens from patients with tolvaptan-induced liver injury.
- The ultimate ambition is to define the role of T-cells in the aetiology of tolvaptan-induced liver injury utilizing in vitro T-cell culture methods.

4.3 Methods.

4.3.1 Study approval.

- Toxicity studies were performed utilising TVP, and TVP metabolites DM-4103 and DM-4107 on PBMCs from healthy donors obtained in Liverpool. Approval for sample collection was acquired from the Liverpool local research ethics committee and informed written consent was obtained.

- Patient blood samples: PBMC samples from a total of nine TVP-exposed patients who displayed signs of hepatic injury were collected from global sites with approval and consent obtained by Otsuka Pharmaceuticals. Cryopreserved PBMC were transferred to Liverpool for functional analysis.

4.3.2 Human subjects and cell isolation/separation.

In this study, we used PBMC samples from a total of 9 tolvaptan-exposed patients who displayed signs of hepatic injury. PBMC from each patient were counted and divided for various uses ($5x10^6$ for EBV-transformed B-cell generation (to act as an immortalised, autologous source of antigen presenting cell), minimum of $3.6x10^6$ for the lymphocyte transformation test, $3x10^6$ for bulk cultures, excess to be frozen). An LTT was performed whereby patient PBMC ($1.5x10^5$/well) were cultured for 5 days with a range of drug-antigen doses within and up to the limits of the non-toxic dose range established by the previously described toxicity assays.

4.3.3 Medium for T-cell culture and cloning.

The medium used is described in chapter 2 section 2.2.

4.3.4 Identifying the drugs optimal dose (Toxicity assay).

Blood samples were collected from healthy donors, PBMCs were isolated and toxicity studies were performed utilising TVP, DM-4103, and DM-4107. PBMCs were exposed to each drug over an initially broad dose range, typically 0.01-1000 μM, in which the likely toxicity range to the vast majority of drugs can be detected for a PBMC population. After 72 hrs, PHA was added to non-specifically stimulate the proliferation of T-cells.

4.3.5 Lymphocyte Transformation Test (LTT).

LTT assay was performed on PBMC isolated from TVP DILI patients using an established protocol. Briefly, PBMC ($1.5x10^5$ cells) were cultured in a 96-well U-bottom cell culture plate in triplicate with either TVP or one of the metabolites and incubated at 37°C, 5% CO_2 for 6 days. In this assay tetanus toxoid (TT) 5 μg/mL was used as a positive control while culture medium was the negative control. 16 hrs prior to harvesting [^{3}H]-thymidine (0.5 μCi/well) was added and lymphocyte proliferation was assessed as counts per minute (cpm) using scintillation counter (Wallac microbeta trilux, PerkinElmer, Cambridge, UK) (Figure 4.1). Proliferative responses (counts per minute [cpm]) were converted to a stimulation index (SI) representing the cpm in drug treated cultures divided by the cpm in medium control.

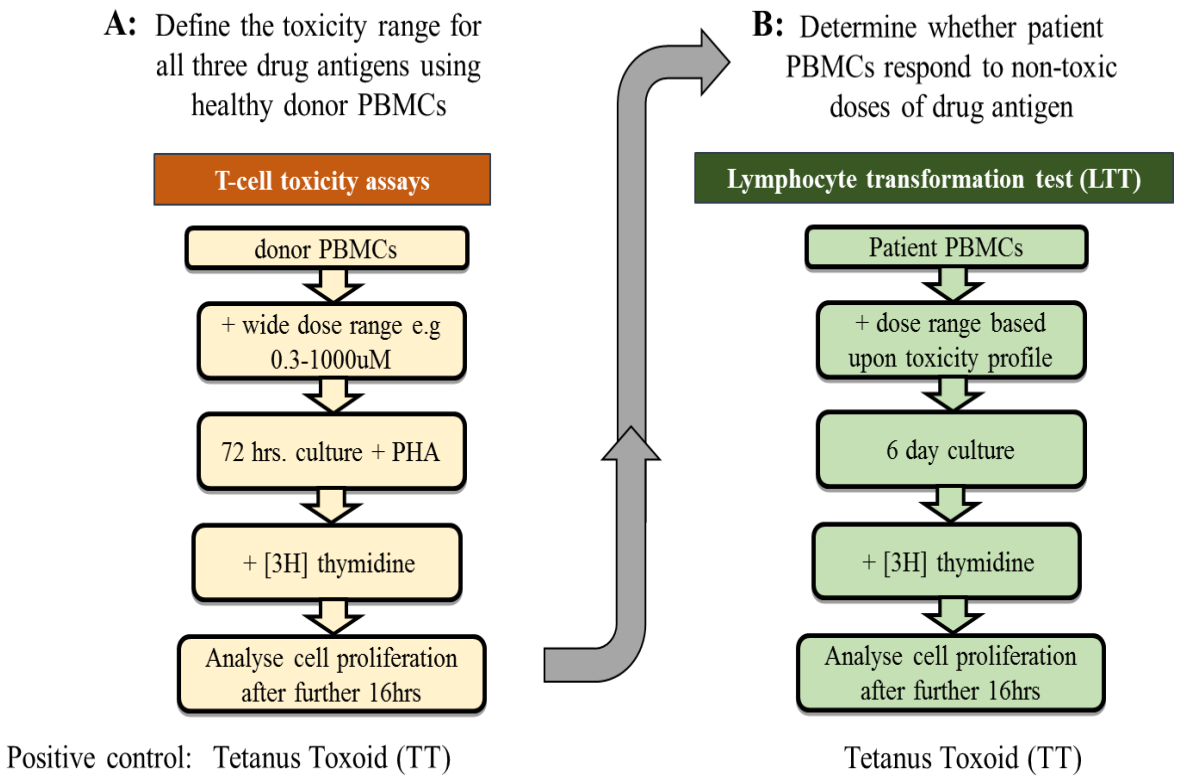


Figure 4-1 A. Toxicity assay to identify the optimal doses for TVP and the metabolites. PBMCs from healthy donors were cultured with a wide range of drug doses for 72 hrs then PHA was added for 24 hrs. [^{3}H]-Thymidine (0.5 µCi/well) was finally added to the culture plate.

B: LTT assay. In this test the PBMC isolated from TVP DILI patients were cultured in triplicate with either TVP or one of the metabolites. Plates were incubated at 37°C, 5% CO_2 for 6 days. In this assay tetanus toxoid (TT) 5 µg/mL was used as a positive control while the culture medium was the negative control. 16 hrs prior to harvesting [^{3}H]-thymidine (0.5 µCi/well) was added and lymphocyte proliferation was assessed as counts per minute (cpm) using scintillation counter.

4.3.6 Generation of EBV-transformed B-cells.

Epstein-Barr virus transformed B-cell lines were generated from PBMC and used as antigen presenting cells in experiments with T-cell clones. The protocol is described in chapter 2 section 2.3.3.

4.3.7 Generation of drug-specific T-cell lines and T-cell clones from PBMCs isolated from tolvaptan DILI patients.

T-cell lines were generated by the bulk method described in chapter 2 section 2.3.4. Briefly, PBMC (1x10^6 /mL) were plated into 48 well plates with an optimal concentration of TVP

(30 µM), DM-4103 (40 µM), or DM-4107 (150 µM) and incubated for 14 days. Cultures were fed with R9 medium supplemented with IL-2 (200 IU/mL) on days 6 and 9. On day 14, a sample of these cultures was frozen, while other cells were subject to serial dilution and mitogen-driven expansion using previously described methods in chapter 2 section 2.3.4.2. T-cell clones were then maintained in medium containing IL-2. IL-2, PHA and irradiated allogeneic PBMC were added every 2 weeks to restimulate the clones (Figure 4.2).

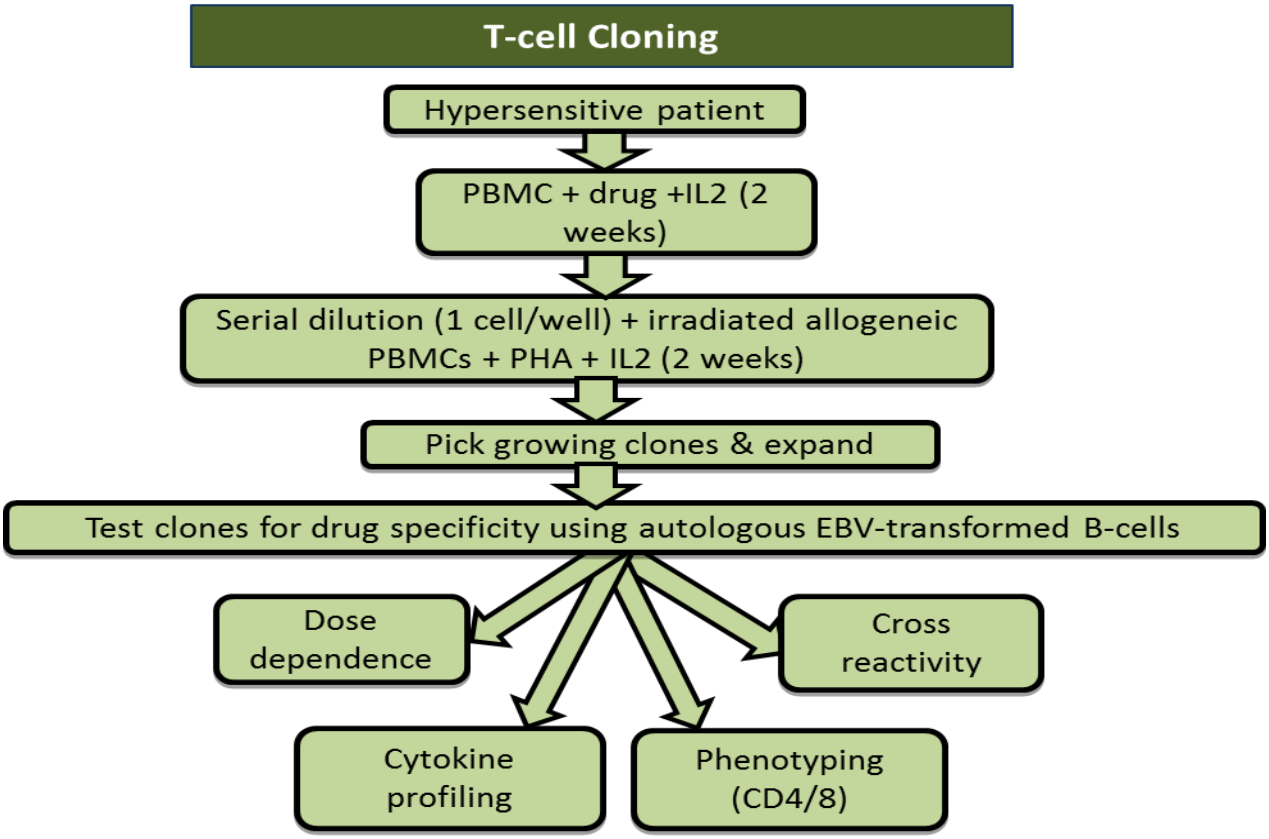


Figure 4-2 T-cell cloning from tolvaptan patients. Scheme depicts the sequence for the events starting with blood collection, generation of single specific T-cells clones, to specific assays to detect the characteristics of the clones.

4.3.8 Initial testing of drug-specific T-cell clones antigen specificity using [^{3}H]-thymidine uptake assay.

After 28 days, picked clones from PBMC treated with TVP, DM-4103 or DM-4107 were expanded to approximately 5x10^4 cells. These clones cultured with TVP (30 µM), DM-4103

(40 µM) or DM-4107 (150 µM) and irradiated EBV-transformed B-cells ($1x10^4$ cells/well) for 48 hrs in two conditions: one containing drug and the other the negative control (medium).

Proliferation was measured by the addition of [^{3}H]-thymidine for 16 hrs followed by scintillation counting. Clones with a stimulation index (SI) (mean cpm drug-treated wells/mean cpm in control wells) equal to or higher than 1.5 were expended for further analysis.

4.3.9 Quantitative and Qualitative assessment of the T-cell clones displaying reactivity to drugs (dose response and cross-reactivity test).

- T-cell clones were incubated with irradiated APCs and a range of concentrations of TVP (10-30 µM), DM-4103 (10-40 µM) and DM-4107 (50-250 µM) for 48 hrs. Proliferation was detected by the addition of [^{3}H]-thymidine.
- The same proliferation protocol was used for assessment of cross-reactivity. However, in this experiment, clones were incubated with TVP and the TVP metabolites. The assays contained a negative (medium) and positive (PHA) controls.

4.3.10 Measurement of cytokine secretion from T-cell clones using ELIspot.

ELIspot analysis for various cytokines was conducted using the protocol described in chapter 2 section 2.3.9 after clones were cultured with irradiated EBV-transformed B-cells and either TVP, DM-4103 or DM-4107.

4.3.11 Characterisation of the phenotypic profile of drug-specific T-cell clones.

Flow cytometry was used to detect expression of specific markers on the surface of T-cells. Phenotypic profiling of T-cell clones followed the same protocol described in chapter 2 section 2.3.11.

4.3.13 Generation of $CD8^+$ T-cells clones.

Previous experience with other drugs, such as flucloxacillin, have highlighted the importance of $CD8^+$ T-cells in the aetiology of drug-induced liver injury, but also the difficulty in generating drug-specific $CD8^+$ T-cell clones as they tend to be less abundant than the $CD4^+$ population. To try and characterise the $CD8^+$ T-cell response, and focussing on DM-4107 as our most promising target to date, we thawed DM-4107 patient 2, 4, 5, 6 bulks and isolated the $CD8^+$ T-cell population using a $CD8^+$ magnetic bead separation kit (miltenyi biotec) and conducted serial dilution experiments.

4.4 Results.

4.4.1. Toxicity test results.

For all three drugs toxicity was observed (Figure 4.3). A sharp reduction in the proliferative response induced by PHA was due to the drug-specific induction of T-cell death. Further, toxicity assays were then performed with more limited dose ranges around the proposed toxic threshold to provide a more defined safe-dose limit. Tolvaptan and DM-4103 induced T-cell death upwards from 40 µM and 50 µM, respectively. DM-4107 was far less toxic, only resulting in toxicity from 400 µM.

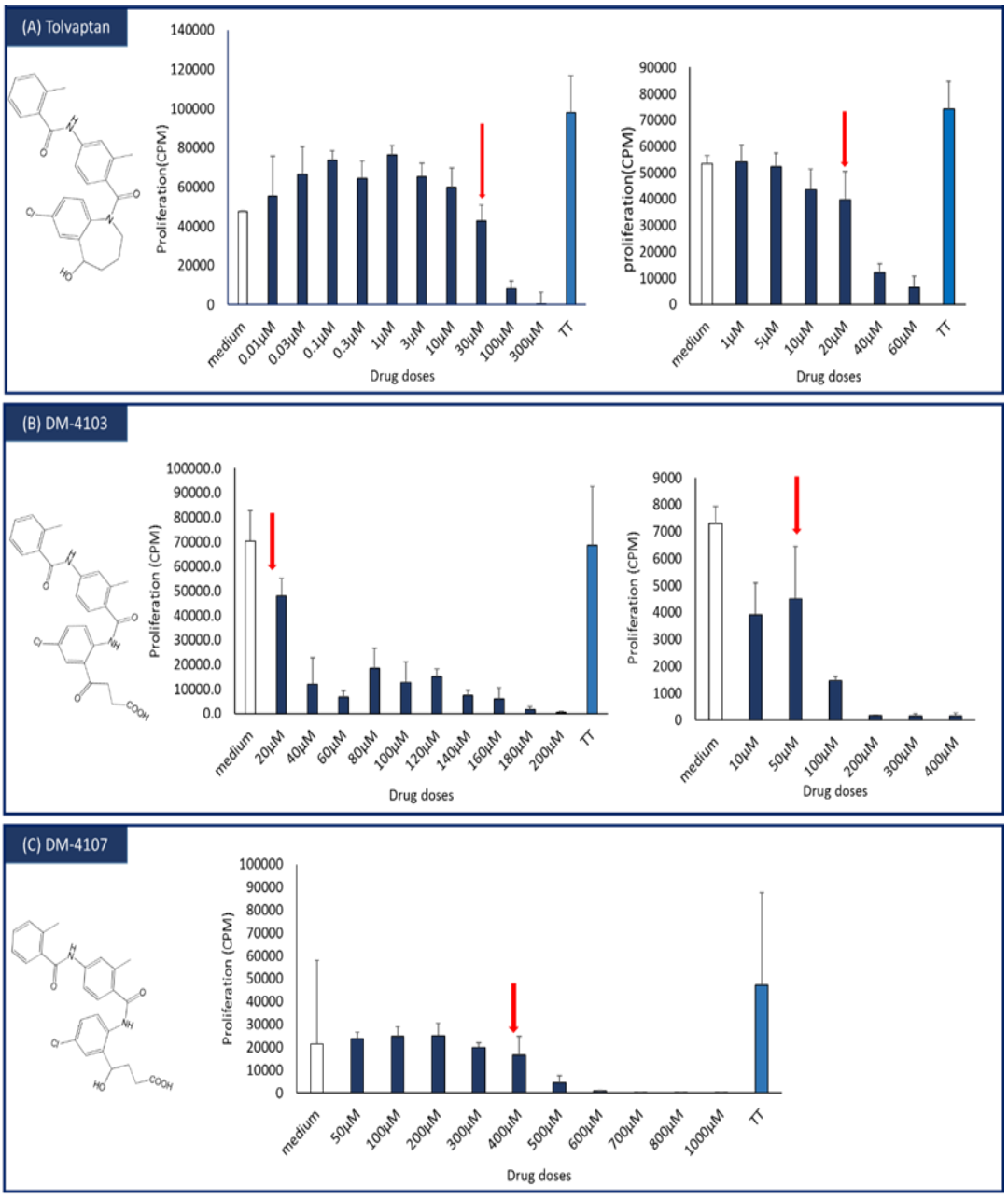


Figure 4-3 Toxicity assay. Healthy donor PBMCs were cultured with drug antigens at varying concentrations for 72 hrs. PHA was then added to stimulate PBMC growth prior to measuring cell death using [^{3}H]-thymidine incorporation. Cells with medium or non-toxic drug doses should proliferate due to PHA, while toxic doses should kill cells and thus have a low proliferative response.

4.4.2 LTT test results.

For all patients, no significant increase in PBMC proliferation was identified in response to TVP, DM-4103, or DM-4107 (Figure 4.4). In contrast, proliferation was observed in

response to the addition of tetanus toxoid (TT; positive control). No TT response was detected for patient 7. While this could have indicated a lack of viable cells, the lack of TT response could also represent a previous lack of exposure to TT through vaccination, which is necessary for TT to function as a positive control. Despite our inability to validate the viability of the PBMCs isolated from patient 7, we did not remove patient 7 from the study and continued to expose PBMCs to drug-antigens in bulk cultures. While for most donors we received a more than an adequate number of viable PBMC ($12.4\text{-}33.6x10^6$), we were only able to recover $2x10^6$ PBMC from patient 9. To perform all of our standard cultures we require a minimum $12x10^6$, so in order not to waste the sample we created an altered method to utilise the smaller sample. For this sample we separated $1x10^6$ PBMC for the LTT ($6.6x10^4$/well), $1x10^6$ for bulk cultures ($3.3x10^4$/well), and $0.5x10^6$ for EBV-transformed B-cell generation ($1x10^5$/well; 96-well U-bottomed plate).

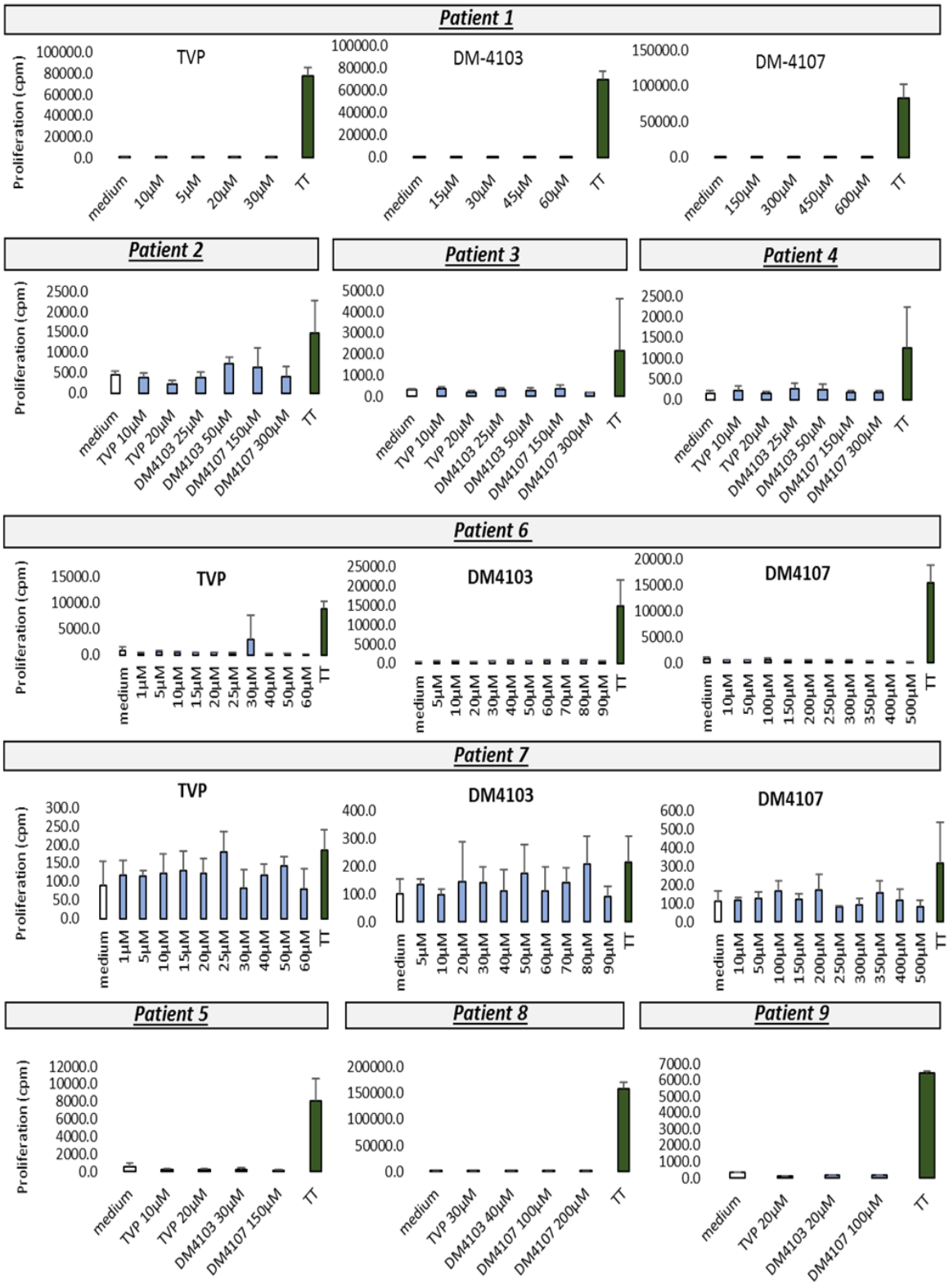


Figure 4-4 TVP-derived antigen-induced proliferation of hypersensitive patient PBMCs. Hypersensitive patient PBMCs ($1.5x10^5$/well) were cultured for 5 days with non-toxic concentrations of tolvaptan (1-60 µM), DM-4103 (5-90 µM), DM-4107 (10-500 µM), or tetanus toxoid (TT; positive control). [^{3}H]-thymidine (0.5 µCi/well) was added and incubated for 16 hrs. Data presented as radioactive counts per minute (cpm) ± SD of triplicate cultures.

4.4.3 Generation of drug-specific T-cell clones.

A handful of clones surpassing the 1.5 stimulation index threshold could be identified (Figure 4.5). The majority of which were responsive to TVP-metabolites rather than tolvaptan itself. For patients 7 and 9, no clones were found to be responsive to any antigen. Patient 7 was the patient whose PBMCs failed to respond to TT in the LTT and thus both assays together indicate low cell viability, while patient 9 was the sample that we recovered a very low number of PBMC from to begin with and thus the lack of response may be due to the lower propensity to discover a low frequency population in a smaller PBMC sample. Additionally, this analysis was unable to be performed for Patient 8 as on multiple occasions, using fresh vials of frozen PBMCs, cultures to generate EBV-transformed B cells for use in antigen-presentation developed infections. We had been made previously aware that there had been sampling processing issues for this patient before transport to Liverpool. After three failed attempts to generate healthy cell lines we had exhausted the frozen PBMC stock and thus were forced to abandon a further investigation of this patient's response.

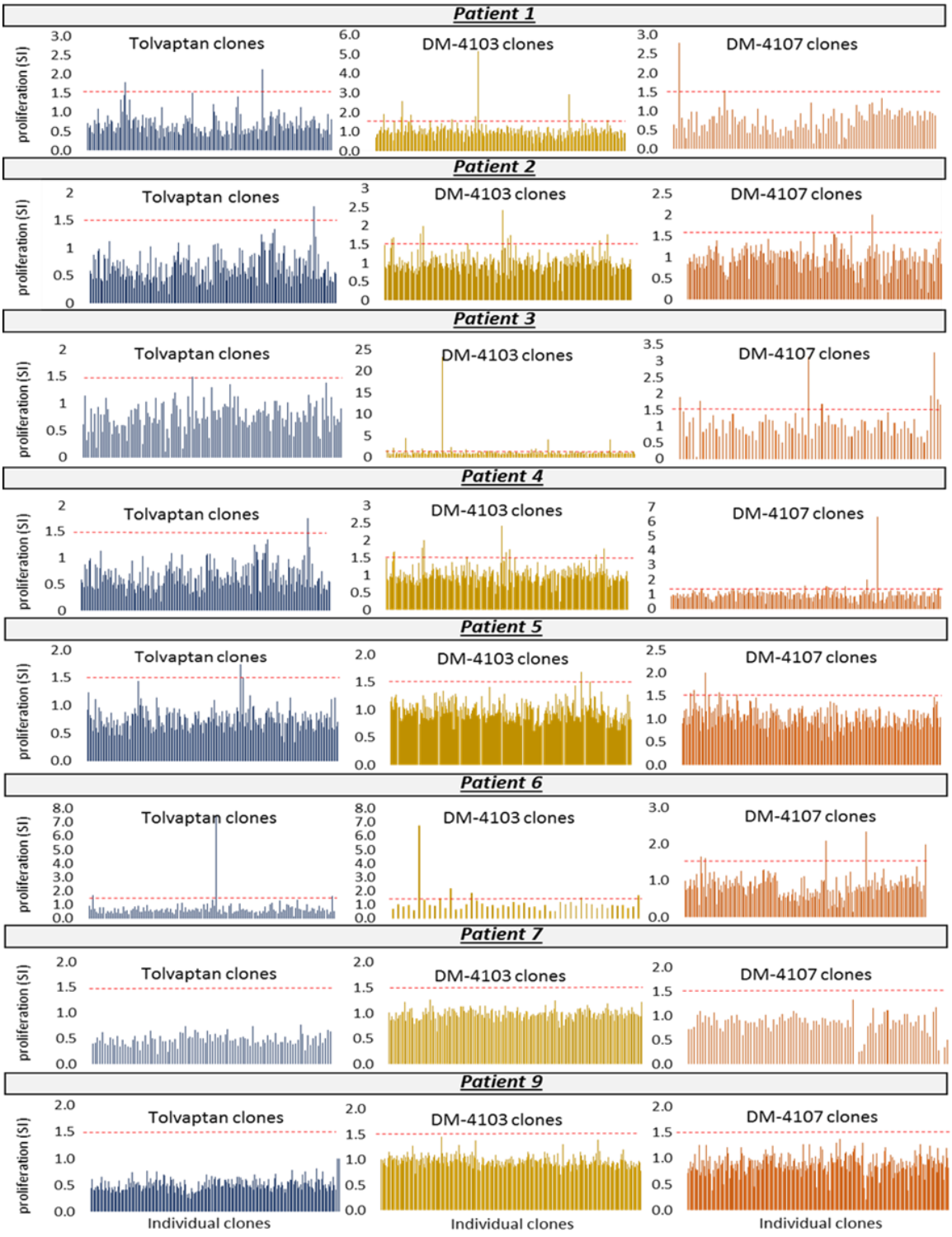


Figure 4-5 Identification of TVP-derived antigen-responsive T-cell clones from hypersensitive patients. T-cell clones ($5x10^4$/well) derived from PBMC cultures with tolvaptan-derived antigens were cultured for 48 hrs with autologous EBV-transformed B-cells ($1x10^4$/well) and the antigen to which they were initially exposed (TVP, 20 µM; DM-4103, 40 µM; DM-4107, 150 µM) or medium alone (negative control). Cells from all patients, apart from patient 8, were subject to duplicate cultures before calculating the stimulation index using average proliferative counts (stimulation index = average of drug-treated wells / average of control treated wells).

4.4.4 Detection of the response of drug-antigen-specific T-cell clones.

Clones which had been deemed antigen responsive in test 1 and expanded were then subject to a more detailed proliferation assay using triplicate cultures to (1) validate the response observed in test 1, (2) to determine dose response using multiple antigen concentrations, and (3) assess cross-reactivity between TVP-derived antigens. For the majority of test 1-responsive clones, test 2 did not show a proliferative response to any drug antigen, (patient 1 figure 4.6), (patient 5 and patient 6 figure 4.7). All clones proliferated strongly in response to PHA (positive control) validating the presence of live cells within each culture. For clones which had expanded more than others after test 1 restimulation, test 2 was also performed using ELIspot to detect secretion of IFN-γ and/or IL-13 in response to antigen. For the majority of clones, the secretion of these cytokines was not enhanced by culture with tolvaptan-derived antigens but was induced by the presence of PHA (Figure 4.8).

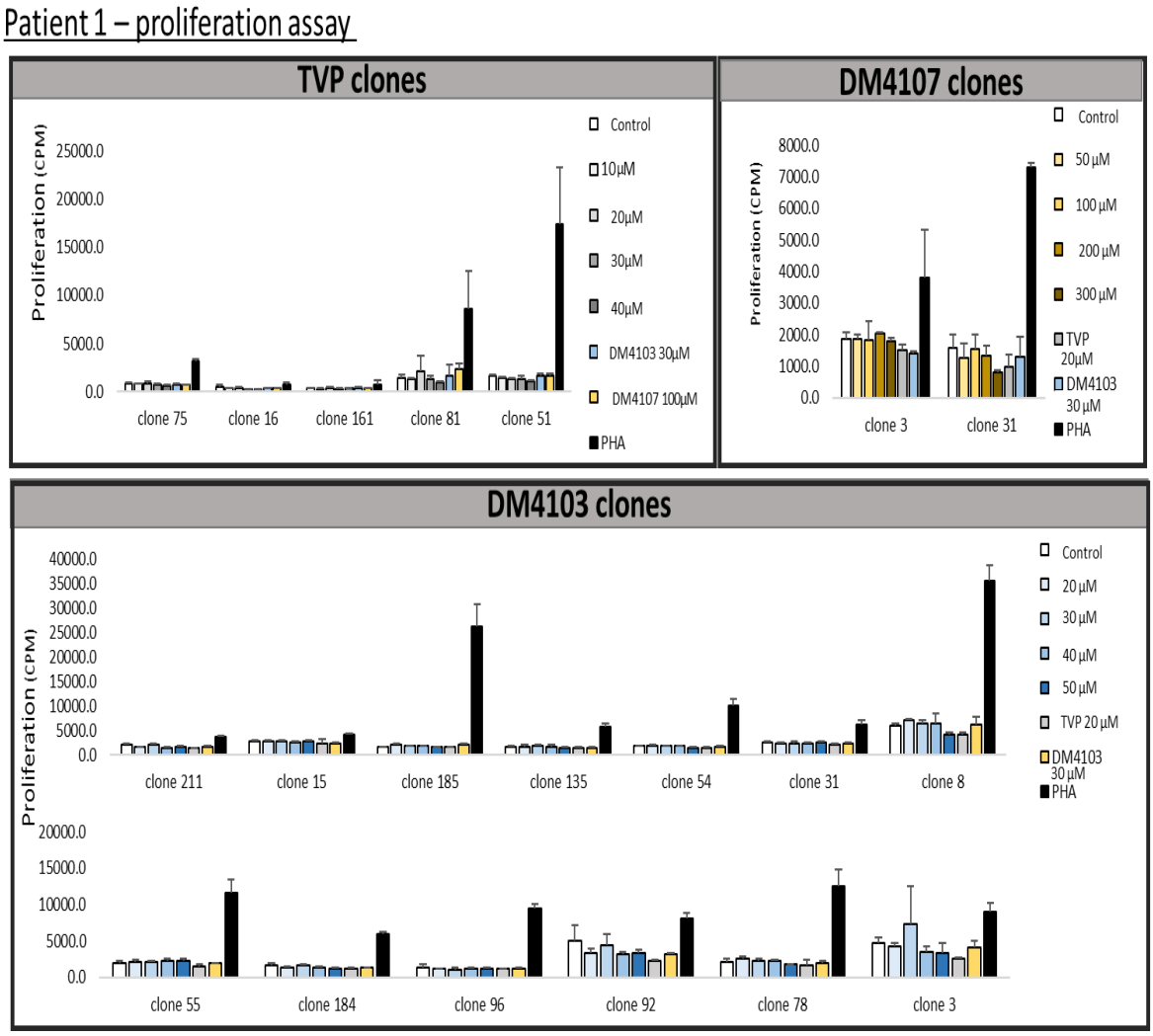


Figure 4-6 Antigen-specific proliferation of TVP and TVP metabolites with cross reactivity for each drug. The specific clones were cultured with irradiated autologus EBVs transformed B cells with drug (TVP, DM-4103 and DM4107) and control condition (without drugs only medium). After culture for 48 hrs in similar conditions as before, proliferation was measured by adding [^{3}H]-thymidine (0.5 µCi/well) for a final 16 hrs incubation. Data representative donors shows the mean ± SD of triplicate cultures.

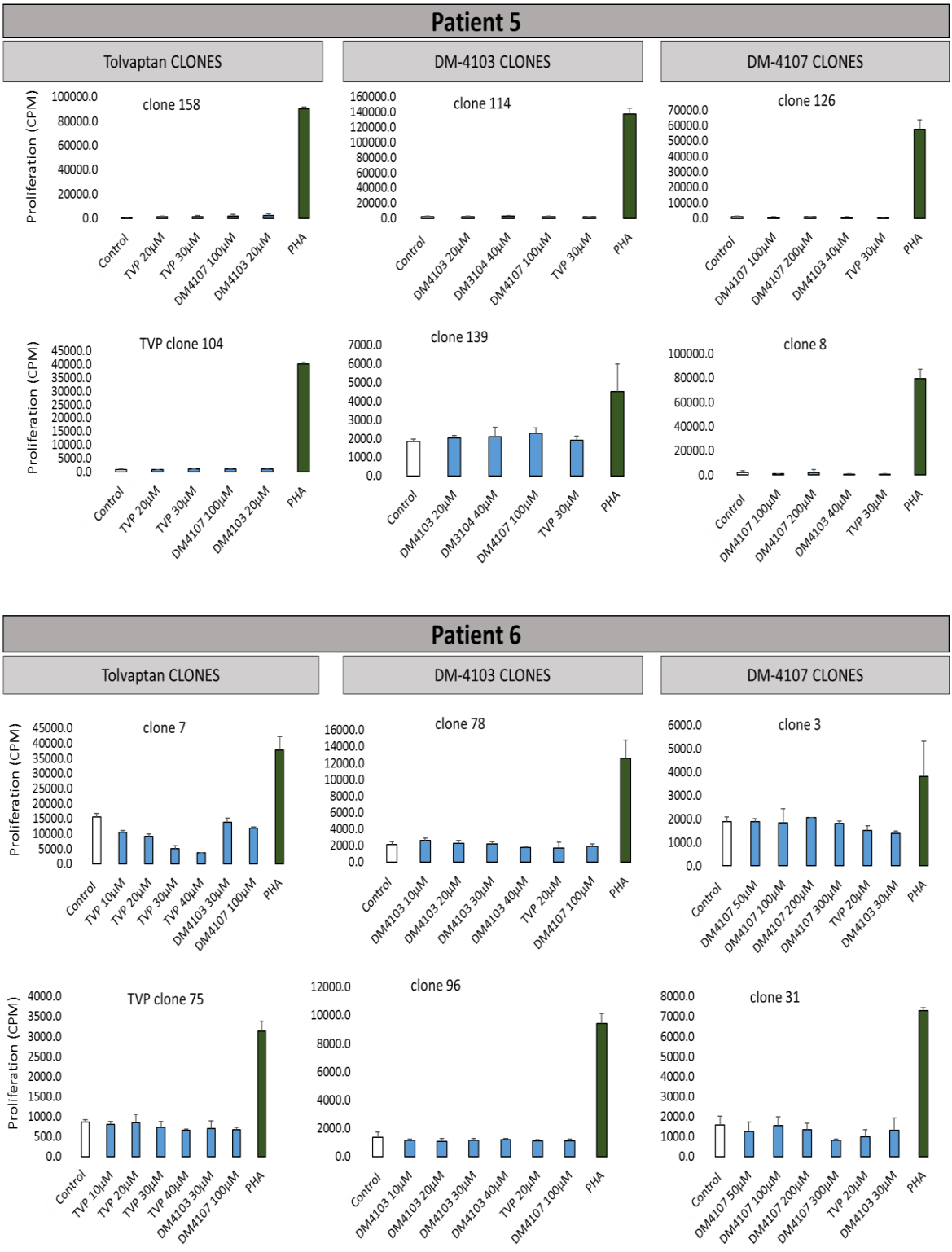


Figure 4-7 Antigen-specific proliferation for the drug-specific clones from patient 5 and 6. The specific clones were cultured with irradiated autologus EBVs transformed B cells with drug (TVP, DM-4103 and DM4107) and control condition (without drugs only medium). After culture for 48 hrs in similar conditions as before, proliferation was measured by adding [^{3}H]-thymidine (0.5 μCi/well) for a final 16 hrs incubation. Data representative donors shows the mean ± SD of triplicate cultures.

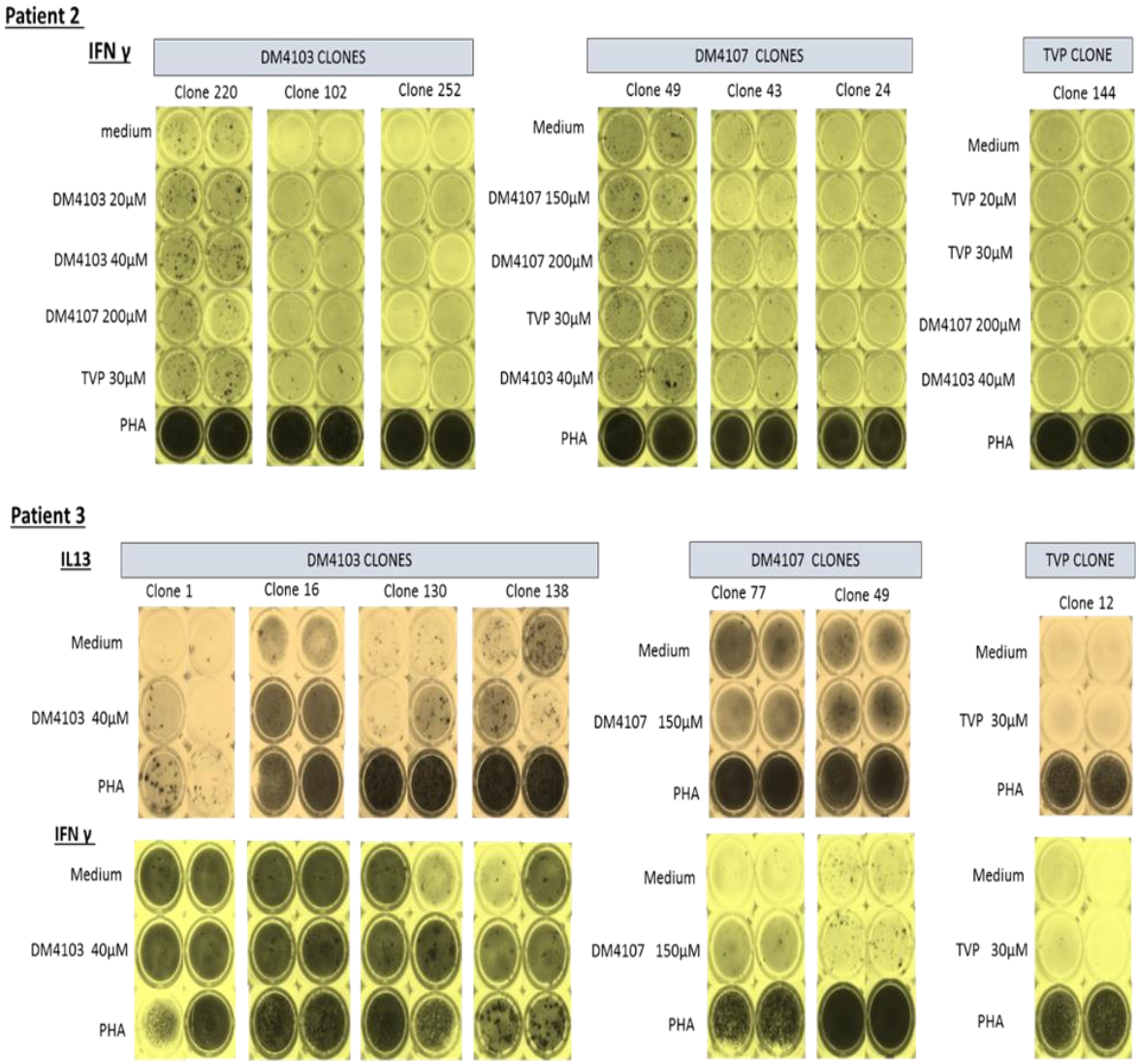


Figure 4-8 Cytokine secretion assay using T-cell clones derived from TVP patients. ELIspot plates were coated with IFN-γ and IL-13 capture antibody and incubated at 4°C overnight. (A) TVP, DM-4103 and DM-4107 specific T-cell clones ($5x10^4$/well; total volume, 200 µL; 96-well ELIspot plate) were cultured with drugs and autologous irradiated EBV-transformed B-cells ($1x10^4$/well) in an atmosphere of 5% CO_2 /37°C for 48 hrs. The ELIspot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELIspot reader.

In contrast, a handful of T-cell clones displayed antigen-specific proliferation and/or cytokine secretion. Clone 120, derived from patient 4 bulk cultures with DM-4107 proliferated in response to DM-4107 (50-150 µM) during tests 1 and 2 (Figure 4.9).

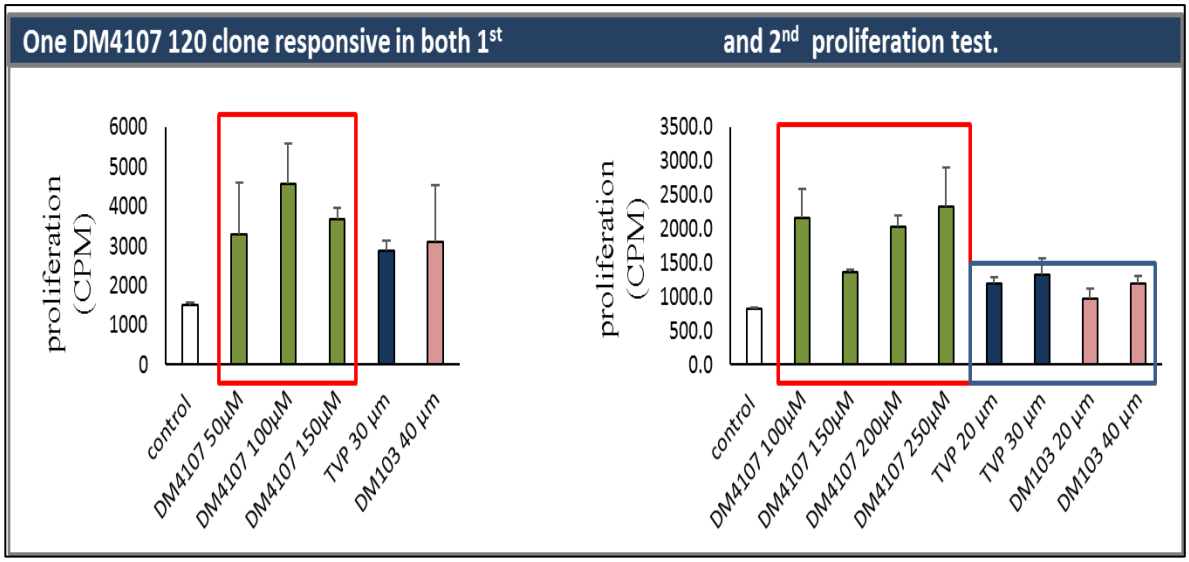


Figure 4-9 [3H]-thymidine proliferation assay for DM-4107-specific T-cell clone 120. T-cell clone (no. 120) was incubated with irradiated autologous EBVs and DM-4107 (50, 100 and 150 µM) in 1st test and the doses were increased in the 2nd test (in range from 100- 250 µM) with added two conditions for TVP 30 µM in 1st test which increased in next one to two doses (20 and 30 µM) and DM-4103 40 µM, in 1st test which increased to 20 and 40 µM in 2nd test. The incubation period for 48 hrs. In the last 16 hrs, [^{3}H]-thymidine was added which was followed by harvesting and reading plate with beta counter. The clone shows a dose-dependent response with both of the proliferation tests. (CPM) represents the count per minute.

Although weak cross-reactivity was indicated with both TVP and DM-4103 in test 1, an IL-13 ELIspot which was simultaneously performed during test 2 clearly indicated strong DM-4107-specific cytokine secretion, alongside a clear lack of cross-reactivity (Figure 4.10 A and B). After restimulation, confirmatory proliferative and cytokine secretion analyses were performed for this clone in test 3. DM-4107 (100-250 µM) induced the proliferation of T-cells, which showed little cross-reactivity to alternate TVP-antigens (Figure 4.10 C). Due to the increased expansion of T-cells, a broader panel of cytokines (IL-13, IFN-γ, IL-22) and cytolytic molecule (GB; granzyme B) were able to be investigated by ELIspot (Figure 4.10 C). DM-4107 (100-250 µM) induced the DM-4107-specific secretion of IFN-γ and GB alongside IL-13. Cross-reactivity with TVP or DM-4103 was not detected, nor was enhanced secretion of IL-22 in response to any TVP-antigen (Figure 4.10 C).

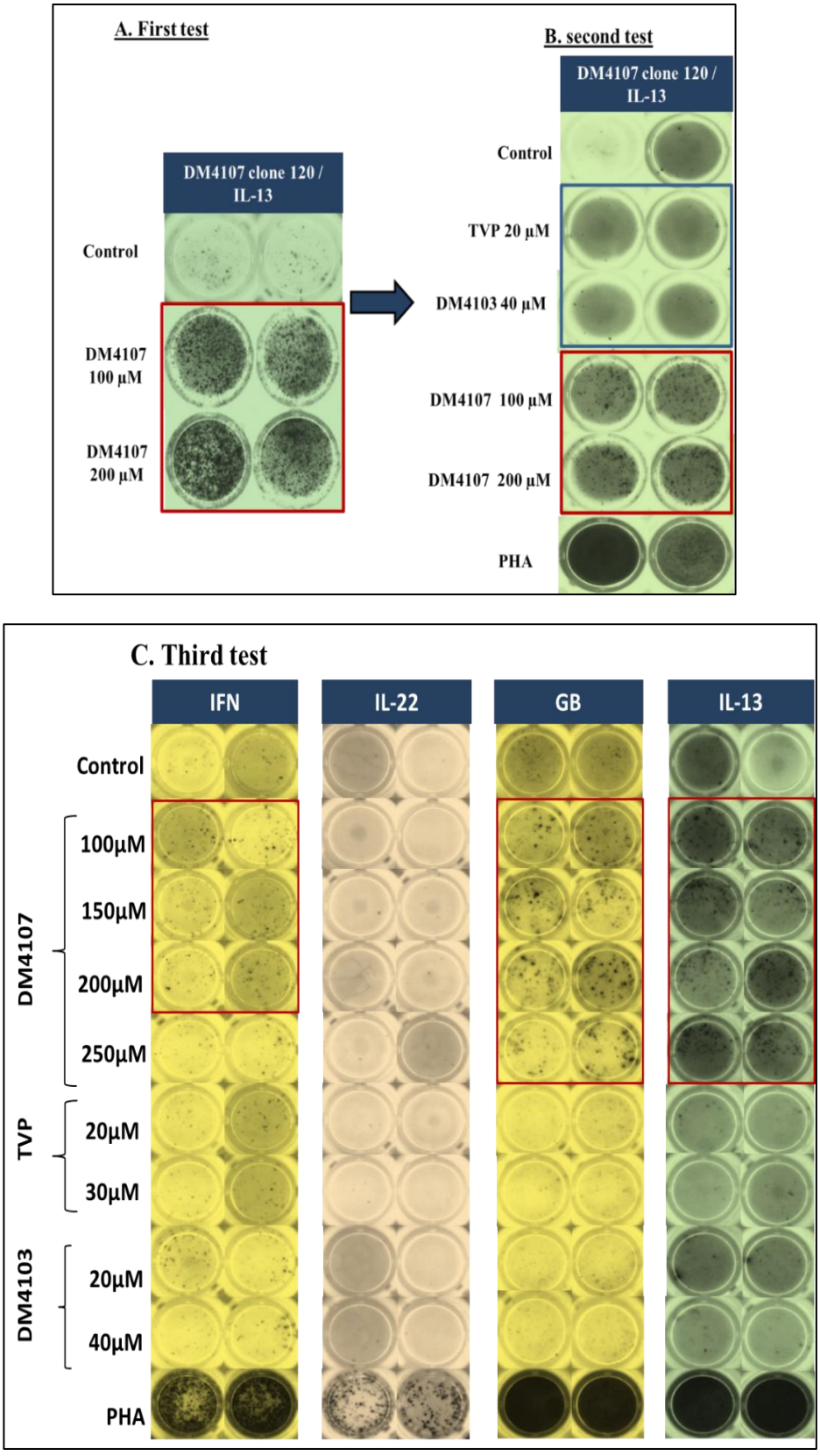


Figure 4-10 (A, B and C): DM-4107 clone 120: initially test for IL-13 secretion using ELIspot. IL-13 secretion in response to both 100 and 200 µM DM4107 was detected with no cross-reactivity to TVP and DM-4103. After this response, a broader range of cytokines were selected (IFN-γ, IL-22, GB and IL-13 again) and the cytolytic molecule granzyme B. DM-4107 exposure resulted in the secretion of IFN-γ, GB and IL-13.

Patient 5 clones 158 and clone 104 which were derived from TVP appeared to show a weak response to the parent drug and the metabolites in the proliferation test (Figure 4.11). Moreover, clone 104 (but not 158) showed low levels of granzyme B secretion in the presence of TVP (Figure 4.13 B).

Clone 52 from patient 5, derived from DM-4107 bulk cultures, also proliferated in response to DM-4107 exposure (100-200 µM) and did not display cross-reactivity (Figure 4.11). Unfortunately, restimulation was unsuccessful and we were therefore unable to generate further data from these clones.

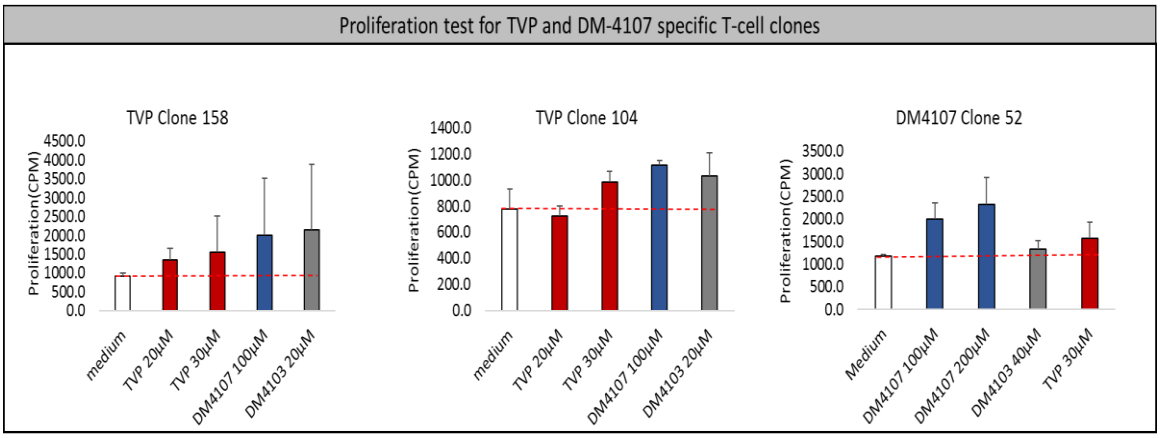


Figure 4-11 Patient 5 proliferation test for TVP and DM-4107. TVP specific (clone 158 and 104) and DM-4107 specific clones ($5x10^4$/well; 96w plate) were cultured with autologous EBV-transformed B-cells ($1x10^4$/well) and an antigen for 48 hrs. [^{3}H]-thymidine was then incorporated cultured for a further 16 hrs before harvesting for analysis of cellular proliferation.

ELIspot assays were performed for a range of patient 5 clones derived from DM-4107 cultures that proliferated in test 1 but not in test 2. A handful of clones secreting low levels of IFN-γ and IL-13 were identified (Figure 4.12 and Figure 4.13). This indicates that T-cell clones responsive to tolvaptan-derived antigens may be more likely to respond by way of cytokine secretion than proliferation. It may well be that the threshold of activation for a proliferative response is set higher than that of cytokine secretion, and only the most sensitive T-cells will mount a proliferative response.

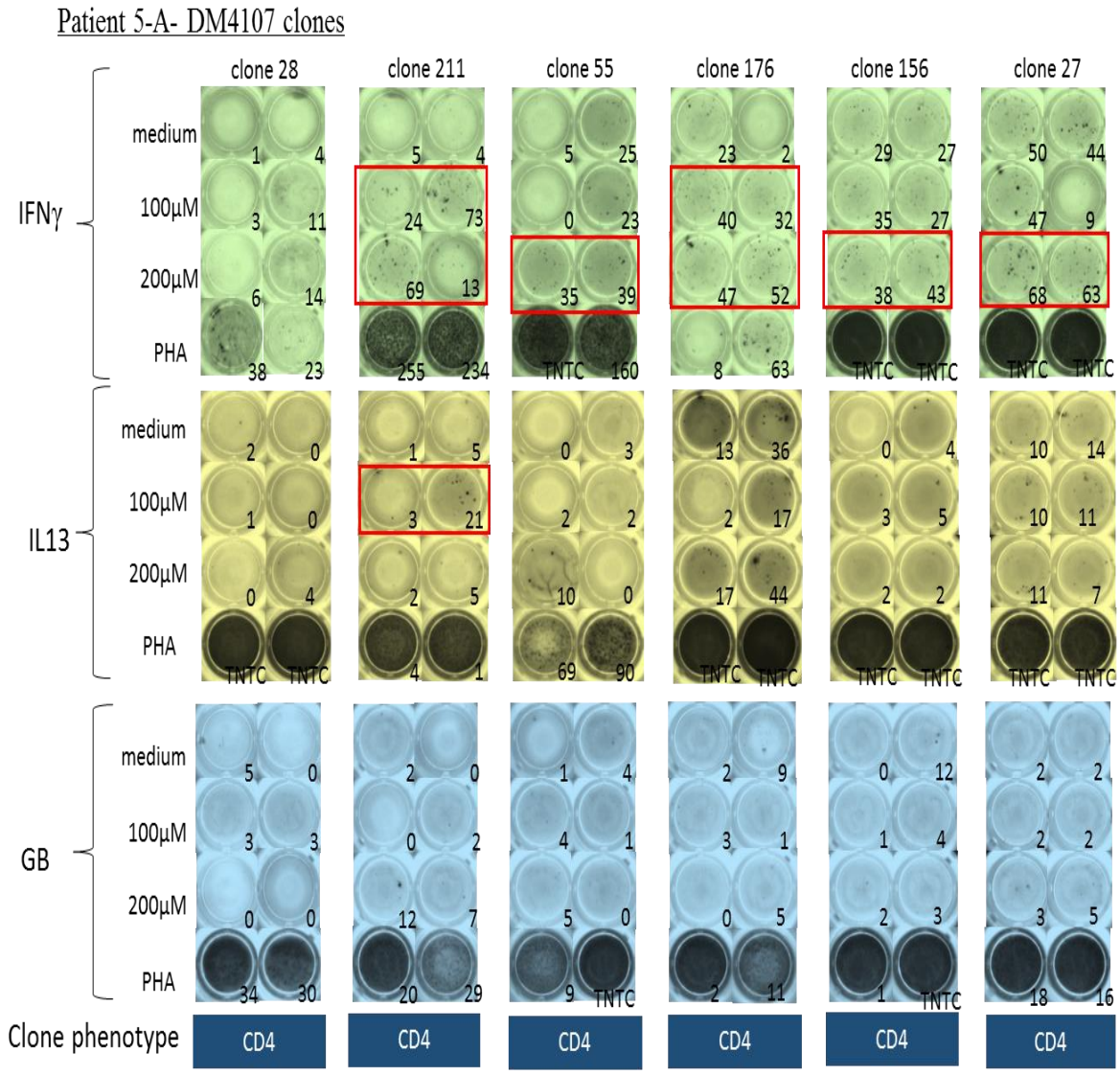


Figure 4-12 A. Identification of cytokine secretion from patient 5 T-cell clones. T-cell clones derived from DM-4107 bulk cultures that did not display a proliferative response during test 2 were subsequently assessed for IFN-γ, IL-13, and granzyme B secretion in response to DM-4107-stimulation (100-200 µM) using ELIspot. T-cell clones ($5x10^4$/well; 96w plate) were cultured on pre-coated ELIspot plates with autologous EBV-transformed B-cells ($1x10^4$/well) and an antigen for 48 hrs. Convincing secretion above baseline is highlighted in red, spot counts are shown for all wells. *TNTC (Too Numerous To Count). Clone phenotype was determined by flow cytometry.

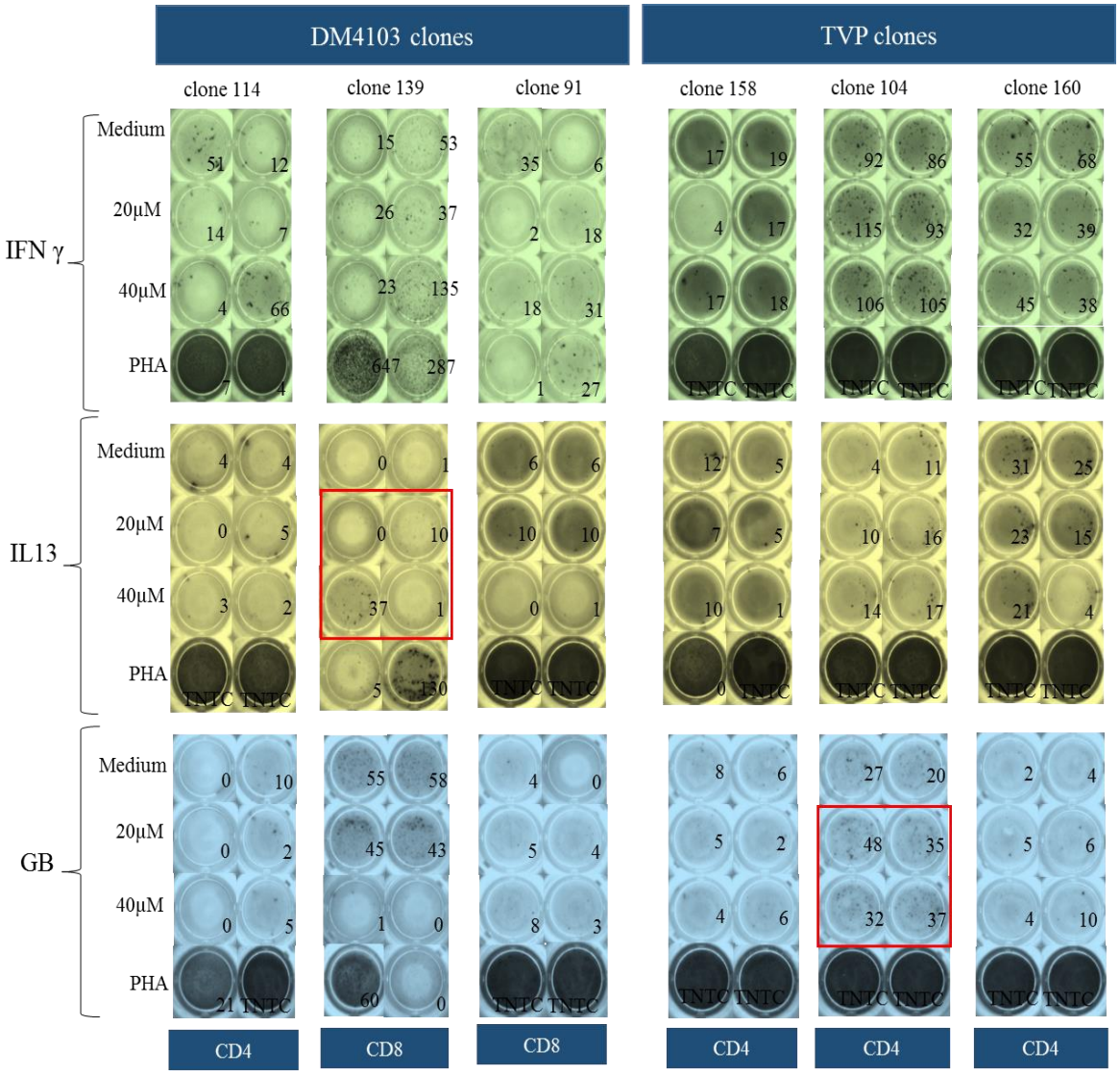


Figure 4-13 Identification of cytokine secretion from patient 5 T-cell clones. T-cell clones derived from TVP and DM-4103 bulk cultures were subsequently assessed for IFN-γ, IL-13, and granzyme B secretion in response to TVP (20-30 μM) and DM-4103 (20-40 μM) stimulation using ELIspot. T-cell clones ($5x10^4$/well; 96w plate) were cultured on pre-coated ELIspot plates with autologous EBV-transformed B-cells ($1x10^4$/well) and an antigen for 48 hrs. Convincing secretion above baseline is highlighted in red, spot counts are shown for all wells. Clone phenotype was determined by flow cytometry.

4.4.5 Detection of antigen-specific $CD8^+$ DM-4107 T-cell clones.

$CD8^+$ T-cell clones were generated from patient 2, 4, 5 and 6 DM-4107 PBMC bulk. The vast majority of clones were not stimulated to proliferate with DM-4107 (Figure 4.14). The small number of clones displaying DM-4107 proliferative responses in test 1 were expended and assessed in dose-response studies using proliferation and cytokines release assays.

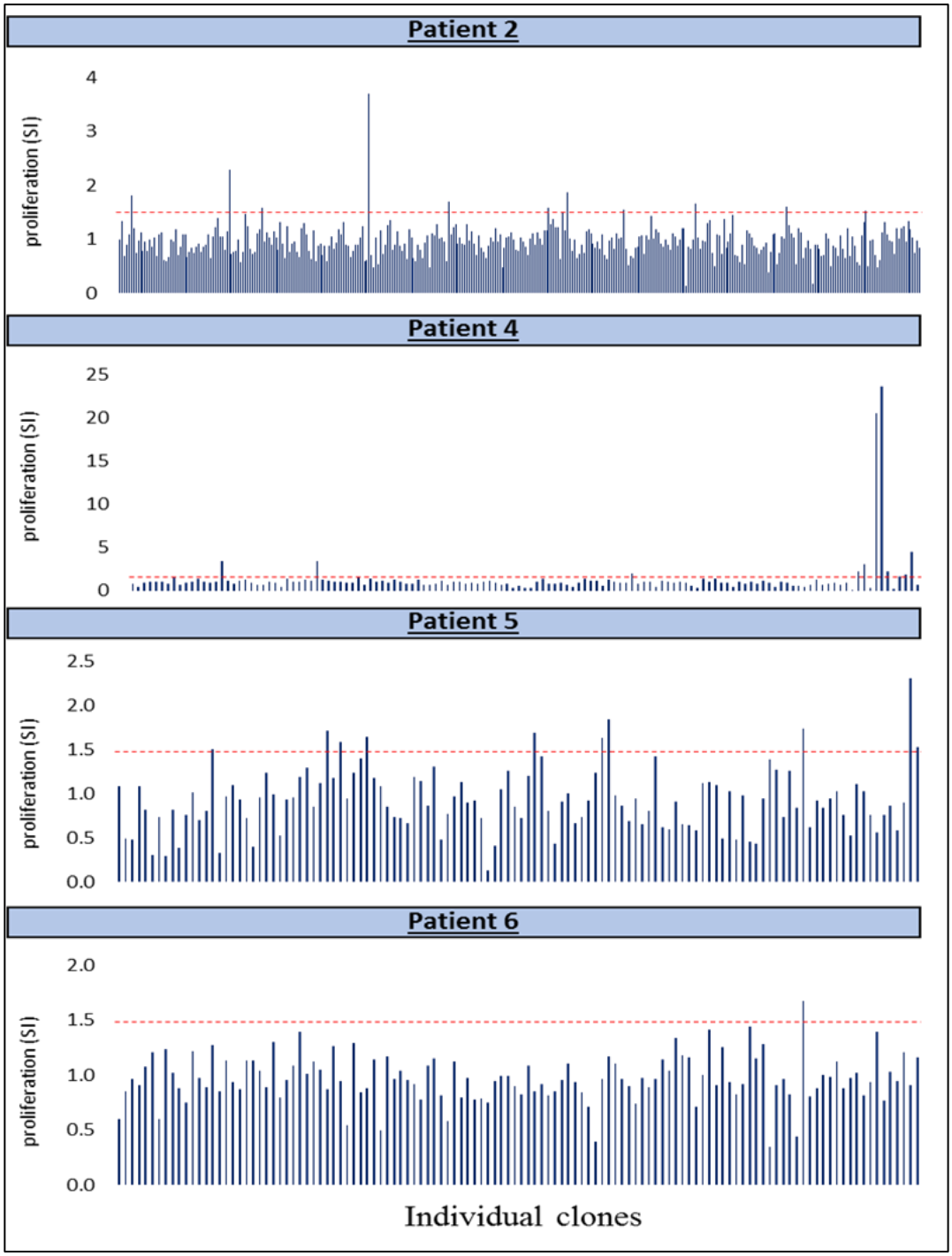


Figure 4-14 Proliferative stimulation of hypersensitive patient CD8$^+$ T-cell clones, derived from DM-4107 treated PBMC. PBMCs (1x10^6/well; 48w plate) were bulk cultured with DM-4107 (150 µM) for 2 weeks before magnetic bead separation of CD8$^+$ T-cells. The CD8$^+$ population was then subject to serial dilution and mitogen-driven expansion to seed and expand an individual T-cell per well. Individual T-cell clones (5x10^4/well; 96w plate) were cultured with autologous EBV-transformed B-cells (1x10^4/well) and an antigen for 48 hrs. [^{3}H]-thymidine was then incorporated and cells cultured for a further 16 hrs before harvesting for analysis of cellular proliferation. Cells were subject to duplicate cultures before calculating the stimulation index using average proliferative counts (stimulation index= average of drug treated wells / average of control treated wells).

High levels of proliferation or cytokine release was not detected in the dose-response studies. Representative INF-γ and IL-13 ELIspot data for patient 5 and patient 6 are shown in figure 4.15.

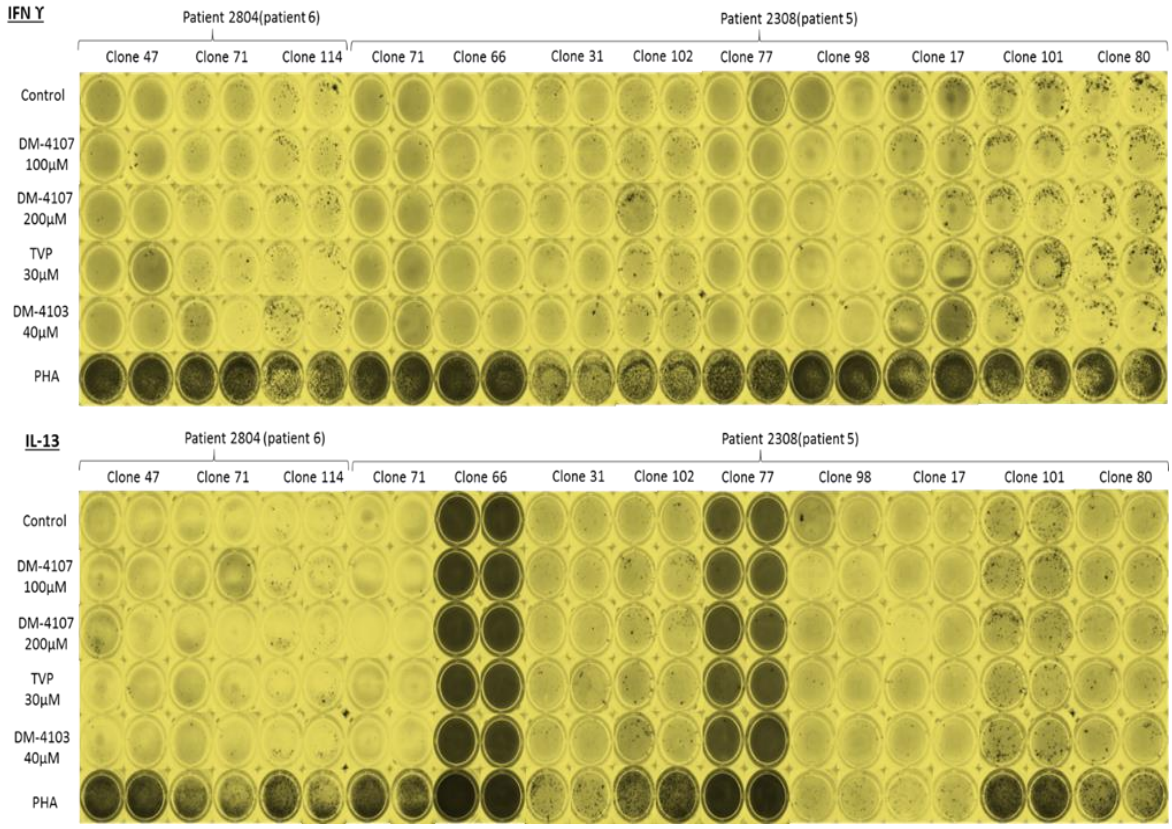


Figure 4-15 Cytokine secretion from patient 5 and patient 6 T-cell clones. T-cell clones ($5x10^4$/well; 96w plate) were cultured on pre-coated ELIspot plates with autologous EBV-transformed B-cells ($1x10^4$/well) and antigen for 48 hrs. In an atmosphere of 5% CO_2 / 37°C. The ELIspot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELIspot reader.

However, a small number of clones displayed weak responses to DM-4107. Patient 4 T-cell clone 16 secreted IFN-γ after exposure to DM-4107, DM-4103 and the parent drug (Figure 4.16 A, B). A similar response was observed with patient 5 clone 106 (Figure 4.17 A, B).

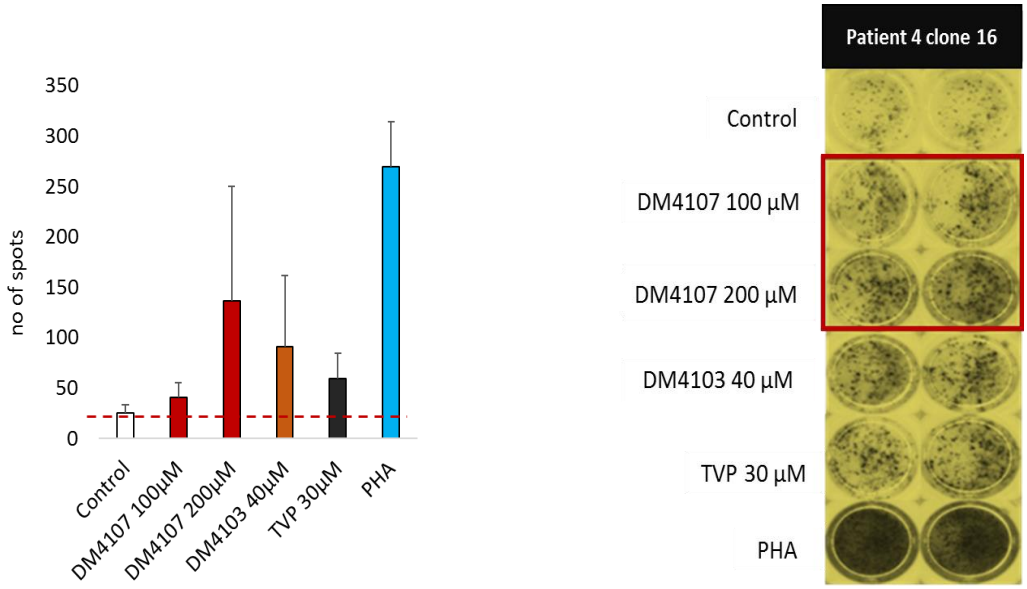


Figure 4-16 Cytokine secretion (IFN-γ) from the DM-4107-specific CD8⁺ T-cell clone from patient 4. T-cell clones ($5x10^4$/well; 96w plate) were cultured on pre-coated ELIspot plates with autologous EBV-transformed B-cells ($1x10^4$/well) and antigen for 48 hrs. In an atmosphere of 5% CO_2 / 37°C. The ELIspot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELIspot reader.

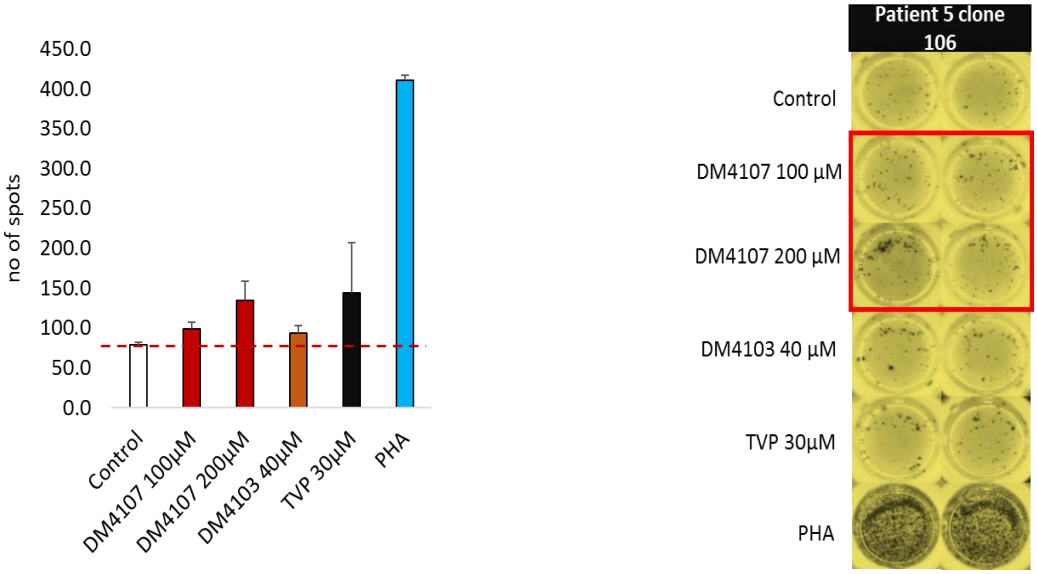


Figure 4-17 Cytokine secretion (IFN-γ) from the DM-4107-specific CD8⁺ T-cell clone from patient 5. T-cell clones ($5x10^4$/well; 96w plate) were cultured on pre-coated ELIspot plates with autologous EBV-transformed B-cells ($1x10^4$/well) and antigen for 48 hrs. In an atmosphere of 5% CO2 / 37°C. The ELIspot plates were washed and developed in concordance with the manufacturer's instructions. Images and SFU counts were analysed from dry wells using an ELIspot reader.

4.5. Discussion.

Delayed-type drug hypersensitivity reactions are mediated by the activation of drug-responsive T-cells, which have been isolated from the blood, skin, and reaction-induced blister fluid of patients with reactions in the skin. Indeed, the role of T-cells in the development of cutaneous drug hypersensitivities are well-defined, however the similar characterisation of those that induce liver reactions has been more difficult, partly due to the comparative inaccessibility to the diseased tissue. Although the skin is the major organ affected by delayed drug hypersensitivity reactions, the liver is the primary internal organ targeted by drug-responsive T-cells, responsible for >9% of ADRs (Lazarou et al., 1998). For many drugs that induce liver reactions, the onset of toxicity has now been associated with the expression of a particular HLA allele (e.g., ximelagatran and HLA-DRB1*07:01 (Monshi et al., 2013c), and nevirapine and efavirenz with HLA-DRB1*01 (Vitezica et al., 2008). Further, flucloxacillin-induced liver injury is associated with HLA-B*57:01, which although not 100% predictive, narrows the affected population to 1 in 500-1000 individuals (Andrews et al., 2010). Importantly, HLA-B*57:01-restricted $CD8^+$ cytotoxic T-cell clones have been identified and characterised from the peripheral blood of patients with flucloxacillin-induced liver injury (Monshi et al., 2013b) providing the foundation for the similar characterisation of T-cells responsive to alternative drugs such as the nonpeptide vasopressin V2-receptor antagonist tolvaptan.

Tolvaptan is used to treat autosomal dominant polycystic kidney disease (AKPKD), the most common monogenic kidney disease and fourth leading cause of end-stage kidney disease in adults worldwide (Torres, 2018). It represents the first pharmaceutical agent to be approved in Europe for delaying the progression of ADPKD in adults with stage 1-3 chronic kidney disease (Blair and Keating, 2015). However, alongside a variety of mild adverse events, it is associated with idiosyncratic elevations of liver enzymes, which are reversible on

discontinuation of the drug. Furthermore, the elevations in liver enzymes progresses to potentially fatal liver injury in a minority of patients. Of interest, DILI only occurs within the AD-PKD patient group and not others treated for alternative indications including cirrhosis, congestive HF, or hyponatraemia, implicating disease pathophysiology in the onset of toxicity (Watkins et al., 2015a). However, injury is hypothesised to occur as a result of an adaptive immune attack on the liver founded on the basis of a rapid recurrence of reactions upon rechallenge, the delayed nature of the initial reaction, and very preliminary associations with HLA alleles. However, T-cells responsive to tolvaptan-derived antigens have yet to be identified. Utilising the peripheral blood of nine patients with suspected tolvaptan-induced liver injury, we sought to identify and characterise T-cells responsive to tolvaptan, or its two major metabolites, DM-4103 and DM4107.

While the lymphocyte transformation test was performed to characterize tolvaptan-specific stimulation of PBMC from patients with liver injury, both drug metabolites (DM-4103, DM-4107) as well as tolvaptan itself failed to induce a proliferative response. Previous studies have shown similar results with drugs associated with T-cell hypersensitivity reactions, where the low frequency of drug antigen-specific responder cells within the peripheral lymphocyte population equates to an undetectable proliferative response within the relatively short term, *in vitro* culture framework of the lymphocyte transformation test (Monshi et al., 2013b). After responder T-cell expansion in bulk cultures with antigen for two weeks, we were able to identify individual T-cell clones that proliferated and secreted cytokines (IFN-γ, IL-13) and the cytotoxic marker granzyme B in response to DM-4107. Importantly this phenotype indicates that these T-cells are pro-inflammatory and capable of inducing cell death through the release of key cytotoxic mediators that may be culpable for damage within the liver. Furthermore, and in agreement with the liver-specific targeting of this reaction, responsive T-cell clones failed to secrete IL-22 which is thought to be important for the

development of cutaneous reactions after release from Th17 and Th22 populations (Eyerich et al., 2010, Akdis et al., 2012, Cavani et al., 2012). Initially only $CD4^+$ T-cells were identified, likely due to the known superior proliferation of these cells over the $CD8^+$ population meaning that when generating clones from bulk cultures, the $CD4^+$ population is more readily expanded. Thus, bulk cultures were revisited and the identification of $CD8^+$ T-cell clones was only achieved after magnetic bead separation of $CD8^+$ T-cells from original bulk cultures. In the $CD8^+$ T-cells, responses were more readily detectable using ELISpot opposed to analysis of cellular proliferation by [^{3}H]-thymidine incorporation. This again corroborates with the reduced likelihood of $CD8^+$ T-cells to proliferate vigorously, when their main objective is to kill the target cells through the secretion of inflammatory and cytotoxic mediators. Interestingly, the DM-4107-specific response lacked cross-reactivity with either DM-4103 or tolvaptan implicating DM-4107 as the immunogenic antigen.

In this study, for the first time we have been successful in identifying and characterising T-cell clones responsive to tolvaptan-derived antigens. T-cell clones were responsive to the DM-4107 major metabolite and lacked cross-reactivity with the parent drug or other major metabolite, DM-4103. However, while well-defined, the metabolism of tolvaptan is extensive with the formation of multiple other metabolites and intermediaries. Thus, while data from this study identifies the capacity of tolvaptan-derived antigens to activate T-cells in patients with associated DILI and provides credence to the original hypothesis that injury is due to an adaptive immune response, it will be the interest of future studies to pinpoint the exact nature of the immunogenic antigen.

Chapter 5: Characterisation of healthy donor-derived T-cell responses specific to telaprevir diastereomers.

5.1 Introduction.

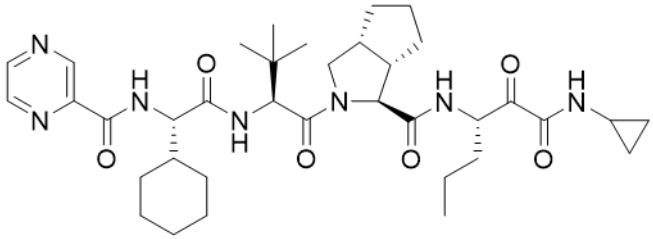
Hepatitis C (HCV) is a serious, potentially life-threatening viral infection that affects an estimated 71 million individuals worldwide (Organization, 2017). Although up to a quarter of infected patients effectively clear the virus (Organization, 2017), the vast majority develop chronic HCV infection. The associated inflammation ultimately leads to severe liver disease, including hepatocellular carcinoma, liver fibrosis and cirrhosis, due to which hepatitis C is the most common indication for liver transplantation in the US (Verna and Brown, 2006b). HCV has been traditionally treated with a dual regimen of PEGylated IFN-α and ribavirin which provide a sustained antiviral response (<10 IU/mL) in just 39% of patients. In contrast, an updated triple treatment regimen including telaprevir (TVR, VX-950), increases the frequency of patients that achieve viral control to 70% (Lang, 2007). Telaprevir is an NS3/4.A protease inhibitor for use against HCV genotype 1 which prevents both the cleavage of viral proteins into active polypeptides for viral assembly, and the deactivation of hepatic cellular proteins essential for mediating the interferon cascade and mounting a viral response (Jesudian et al., 2012, Morikawa et al., 2011, Smith et al., 2011). While administered orally as a single S-configurated diastereomer, telaprevir spontaneously forms the corresponding R-diastereomer (Figure 5.1 A, B), which is approximately 30-fold less pharmacologically active (Garg et al., 2012).

Despite enhanced viral suppression, the triple regimen is associated with an increased risk of adverse cutaneous reactions, with triple telaprevir-containing therapy causing a severe rash in 4.8% of patients compared to just 0.4% with the standard dual therapy. Of more concern, a small subset of patients treated with telaprevir develops life-threatening cutaneous drug hypersensitivity reactions including drug rash with eosinophilia and systemic symptoms (DRESS) and Stevens-Johnson syndrome (SJS) (Pavlos et al., 2012, Roujeau, 2005, Roujeau et al., 2013a). These clinical diagnoses, alongside the lack of correlation

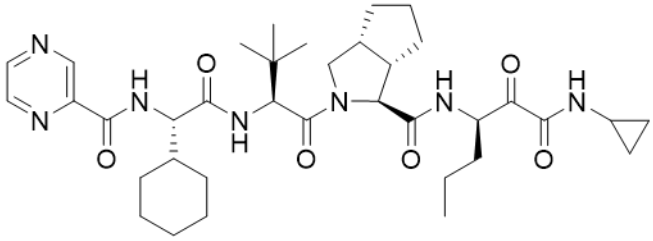
between the severity of telaprevir-induced cutaneous reactions and drug plasma concentration, as well as the delayed onset (median 15 days) and slow resolution (median 44 days) after drug discontinuation, are indicative of a type IV hypersensitivity reaction (Roujeau et al., 2013a). Such delayed drug hypersensitivity reactions are thought to be mediated by the activation and subsequent cytotoxic action of drug-specific T-cells, which have been previously isolated from patients with hypersensitivity to a diverse array of drugs (Kim et al., 2015, Lichtenfels et al., 2014, Meng et al., 2017, Usui et al., 2017a). Despite a reported correlation between the level of the T-cell-derived cytotoxic mediator granulysin release and the severity of telaprevir-induced skin reactions (Suda et al., 2015), telaprevir-specific T-cells have not been identified. Furthermore, no specific HLA alleles are associated with telaprevir-induced skin reactions of any severity.

To circumnavigate the inability of animal models to predict hypersensitivity, *in vitro* models that utilise T-cells from healthy human donors have been developed that are successful at generating and characterising drug-specific T-cells (Bell et al., 2013, Gibson et al., 2017, Monshi et al., 2013a, Sullivan et al., 2018). Critically, these assays enable the modulation of reported susceptibility factors and the identification of the antigen, whether parent compound or metabolic derivative, responsible for the initial, highly-regulated activation of T-cells. telaprevir undergoes extensive hepatic metabolism and forms a range of metabolites, including M11, which was identified as potentially immunogenic due to the induction of a positive guinea-pig maximization test (Figure 5.1C) (Garg et al., 2012). In order to provide an understanding of telaprevir immunogenicity, we utilised *in vitro* peripheral blood mononuclear cell (PBMC) drug bulk cultures to assess the propensity for telaprevir-derived antigens to activate T-cells isolated from drug-naïve healthy human donors.

(A) Telaprevir (S-diastereomer)



(B) R-diastereomer



(C) M11 metabolite (VRT-841125)

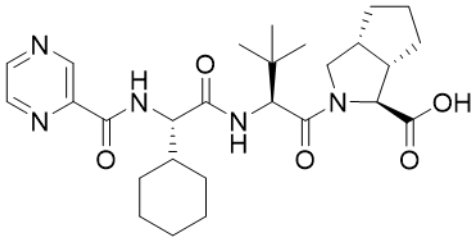


Figure 5-1 Chemical structures of the (A) S- (therapeutic form) and (B) R-diastereomers of telaprevir and (C) the M11 metabolite.

5.2 Materials and methods.

5.2.1 Isolation of PBMC from drug-naïve healthy human donors:

Venous blood samples (120 mL) were taken from seven telaprevir-naïve healthy human donors who had provided informed written consent as directed by the Liverpool local research ethics committee. A density gradient separation technique was performed to isolate the PBMC population from whole venous blood using lymphoprep (Axis-shield, Dundee).

5.2.2 PBMC bulk culture.

PBMCs ($1x10^6$/well; 48-well plate; 660 µL total) were cultured for 14 days with either the S- or R-diastereomer of telaprevir (5-20 µM), or the M11 metabolite (20 µM). Cultures were fed with R9 medium (RPMI 1640, 100 µg/mL penicillin, 100 IU/mL streptomycin, 25 µg/mL transferrin, 10% human AB serum [Innovative Research], 25 mM HEPES buffer, 2 mM L-glutamine) supplemented with IL-2 on days 6 and 9. On day 14, cultures for the same antigen but of differing concentrations were harvested and pooled. A sample of PBMCs were frozen for later use at $10\text{-}20x10^6$ cells/mL at 1:1 ratio of R9 medium to 80% human AB serum, 20% DMSO (total volume, 1 mL). Cryovials were stored at -80°C for 24-48 hrs before transfer to -150°C for longer term storage. Any remaining PBMCs were used for functional studies. Briefly, $1x10^5$/well antigen-exposed PBMCs were re-exposed in duplicate wells (96-well plate, 200µL total) to either the S- or R-diastereomer of telaprevir for 48 hrs (37°C/5% CO_2). Antigen re-exposed cultures were then pulsed with [^{3}H] thymidine (0.5 µCi/well) and subject to a further 16 hrs incubation before analysis of incorporated radioactivity as a measure of drug-specific proliferation using a Microbeta Trilux 1450 LSC beta counter (PerkinElmer, Cambridge, U.K.).

5.2.2 Serial dilution and T-cell cloning.

T-cell clones were generated from PBMC bulk cultures using serial dilution and mitogen-driven expansion (Mauri-Hellweg *et al.*, 1995). Briefly, cells were plated at 1, 3, 0.3 cell/well (96 well U-bottomed plate) in a restimulation cocktail ($5x10^4$ irradiated allogeneic PBMC/well, 10µL/mL PHA, 5µL/mL IL-2) and cultured for 14 days (37°C/5% CO_2). Cultures were fed on day 5 and then every two days subsequently with R9 medium supplemented with IL-2. Additionally, autologous EBV-transformed B-cells (EBV) were generated from PBMC to function as an immortalised antigen presenting cell line.

To probe for antigen-specificity, expanded T-cell clones ($5x10^4$/well; 96 well plate; total volume, 200 µL) were cultured (37°C/5% CO_2) in duplicate per experimental condition with irradiated autologous EBVs ($1x10^4$/well) ± the S- or R-diastereomer of telaprevir (10 µM) or the M11 metabolite (20 µM). After 48 hrs, [^{3}H] thymidine was added before a further 16 h culture prior to analysis of cellular proliferation by scintillation counting. T-cell clones with a stimulation index (mean cpm drug-treated wells/mean cpm of control wells) of >1.5 were repetitively stimulated with allogeneic PBMCs ($5x10^4$/well; 96 well plate; total volume, 200 µL) in R9 medium supplemented with PHA (10 µg/mL) and IL-2 (400 IU/mL) for further expansion.

5.2.3 T-cell clone characterisation assays.

Those clones that responded to telaprevir-derived antigens in a second confirmatory proliferation assay were further expanded and characterised. To define cross-reactivity between telaprevir diastereomers and the M11 metabolite, T-cell clones ($5x10^4$/well; 96 well plate; total volume, 200 µL) were cultured (37°C/5% CO_2) in triplicate with autologous irradiated EBVs ($1x10^4$/well) and either diastereomer (5-20 µM) or M11 (20 µM) for 48 hrs prior to proliferative analysis as described above. In order to explore the requirement for antigen uptake and processing, EBVs were pulsed with telaprevir for 1-16 hrs. After the allotted exposure period, drug-exposed EBVs were washed in PBS and used to restimulate cells as above in the absence of soluble drug. Alternatively, to determine whether antigen was presented in the context of MHC, EBVs were first pre-cultured with either MHC class I or II blocking antibodies or their corresponding isotype controls (5µL; BD Biosciences, Oxford, UK) for 30min. MHC blocked EBVs were then washed and included in the proliferation assay.

ELIspot was used to characterise the drug-specific release of specific cytokines and cytolytic molecules from T-cell clones. The release of IFN-γ, IL-13, IL-22, and granzyme B in

response to telaprevir-derived antigens was visualised by the ELIspot procedure discussed in chapter 2 section 2.3.9 (Mabtech, Nacka Strand, Sweden). Flow cytometry was utilised to characterise T-cell clone phenotype, including clone $CD4^+$ or $CD8^+$ coreceptor expression, to assess the expression profile for a defined chemokine receptor panel (CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CCR9, CCR10, E-cadherin, CLA, CXCR3, CXCR6), and to determine TCR Vβ protein expression using the IOTest Beta Mark TCR Vβ repertoire kit (Immunotech, Beckman Coulter, UK). Briefly, aliquots of T-cells were stained with fluorescence-conjugated antibodies before incubation on ice in the dark for 20 min. Cells were then washed with PBS and resuspended in 200 µL 10% FBS/PBS prior to data acquisition (minimum $5x10^4$ events) using a FACS CANTO II flow cytometer. Data was analysed using FACS DIVA or Cyflogic software (CyFlo Ltd., Finland).

5.3 Results.

5.3.1 Weak telaprevir-specific proliferative response from healthy donor-derived T-cell cultures.

PBMC bulk cultures established with either the S- or R-diastereomer of telaprevir were restimulated every 2-3 weeks to promote further expansion of antigen-specific T-cells. Cultures with high cell recovery were tested for antigen specificity before being subject to serial dilution for T-cell cloning. While responses to the model drug immunogen nitroso sulfamethoxazole (SMX-NO) were clearly detectable from PBMC bulk cultures (Figure 5.2) in all donors, the majority of cultures exposed to telaprevir diastereomers failed to proliferate (Figure 5.2 i and ii; representative donors 1-3). However, T-cells from donor 2 responded weakly in response to the R-diastereomer.

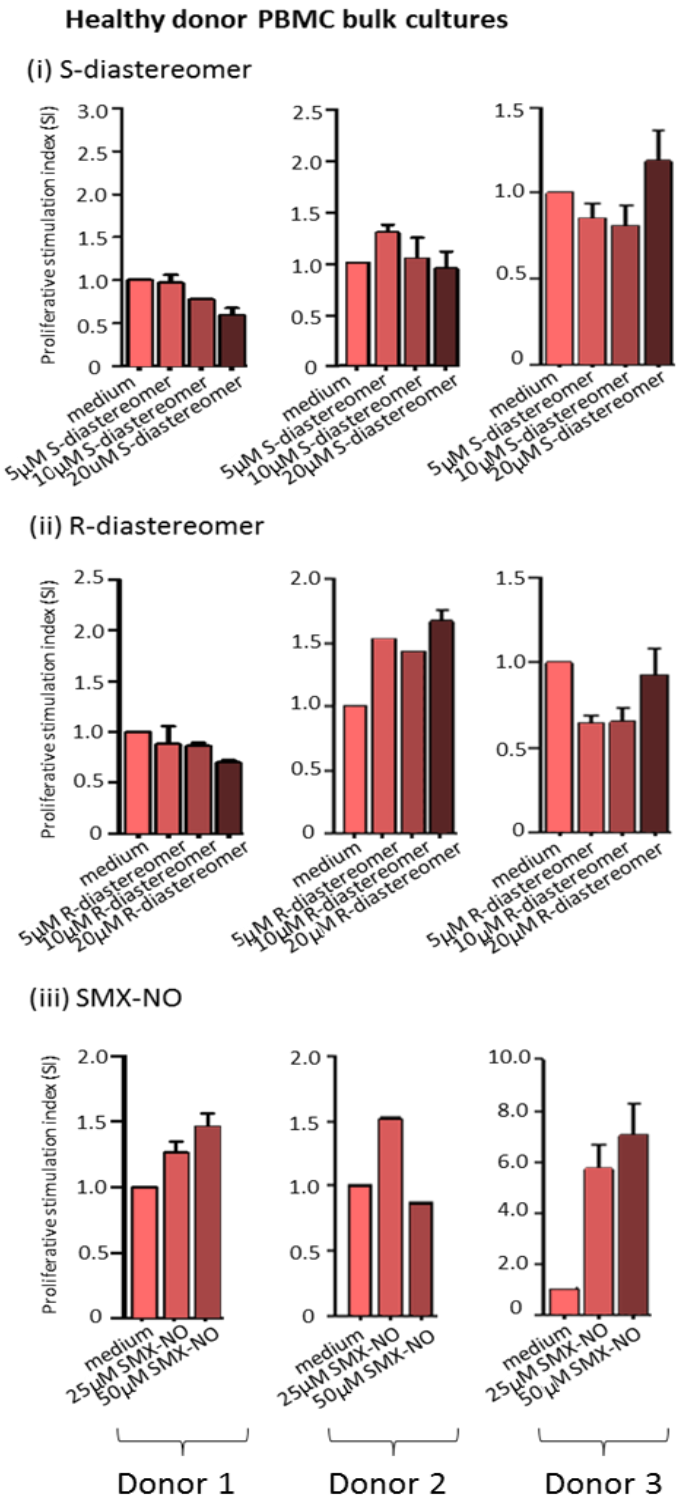


Figure 5-2 Healthy donor T-cell responses from PBMC bulk cultures with the telaprevir (i) S-diastereomer (ii) R-diastereomer (5-20 μM), or (iii) SMX-NO (25-50 μM; model drug-derived immunogen). PBMCs ($1x10^6$/well; 48-well plate; 660 μL total) were directly cultured with antigen for 14 days prior assessment of drug specificity. [3H] thymidine (0.5 μCi/well) was added for the final 16 hrs of the incubation and then incorporated radioactivity was measured. Data shown as proliferative stimulation index (SI; average of drug-exposed wells/average of control wells). Error bars indicate standard deviation for the average of replicate cultures.

5.3.2 Identification of $CD4^+$ and $CD8^+$ telaprevir antigen-specific T-cells.

To explore whether telaprevir-specific T-cells are present below the limit of detection in the PBMC assay, T-cell cloning was performed on cells derived from all seven telaprevir-exposed cultures. Initial testing identified the drug-specific proliferation (SI >1.5) of T-cell clones from 3/7 donors (Figure 5.3).

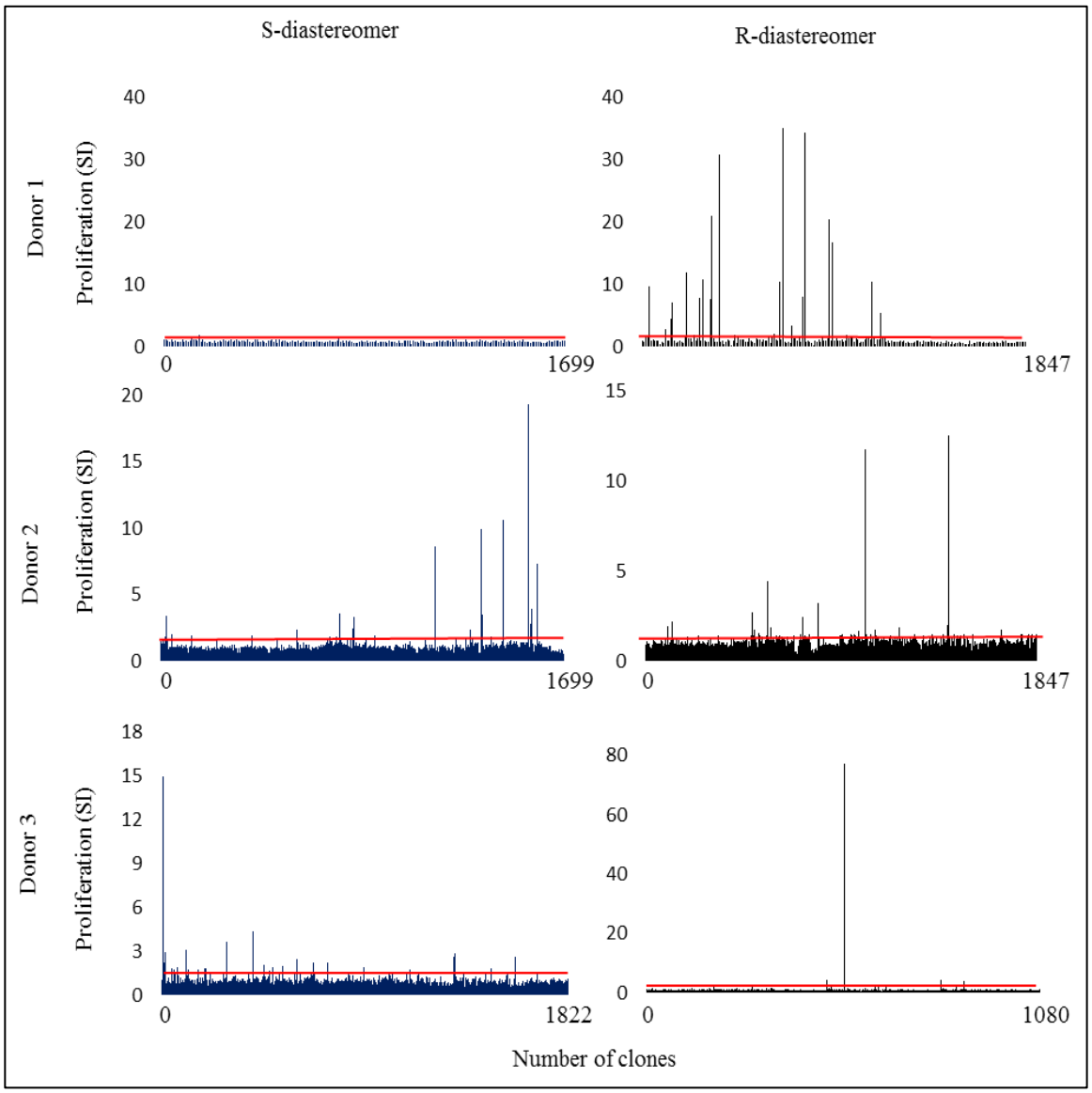


Figure 5-3 Generation of telaprevir-responsive T-cell clones. Telaprevir diastereomer-exposed T-cells from PBMC bulk cultures were subject to serial dilution and mitogen-driven expansion. Individual T-cell clones ($5x10^4$/well; 96 well plate; total volume, 200 µL) were cultured (37°C/5% CO2) in duplicate per experimental condition with irradiated autologous EBVs ($1x10^4$/well) ± the drug antigen. After 48 hrs, [3H]-thymidine was added before a further 16 hrs culture prior to analysis of cellular proliferation by scintillation counting. T-cell clones with a stimulation index (mean cpm drug-treated wells/mean cpm of control wells) of >1.5 were selected as drug-responsive and subject to further expansion and investigation.

After expansion, a further triplicate proliferation culture confirmed the presence of telaprevir-responsive T-cell clones. Five and thirty five T-cell clones derived from PBMC cultures containing the S- and R-diastereomer respectively remained drug-responsive during repetitive mitogen-driven expansions and were used for the mechanistic studies described below.

Irrespective of the antigen used for initial culture, all clones proliferated in the presence of either diastereomer to a similar extent at similar concentrations (Figure 5.4). Flow cytometry determined that all telaprevir diastereomer-responsive T-cell clones from donor 3 were $CD8^+$ T-cells, while a mixed phenotype was observed from the 13 suitable for analysis from donor 2, with 10 $CD4^+$ (76.9%) and 3 $CD8^+$ (23.1%) T-cell clones identified (Figure 5.5).

In stark contrast, cloning performed on PBMC bulk cultures with the M11 metabolite from three healthy donors failed to identify M11-responsive T-cell clones.

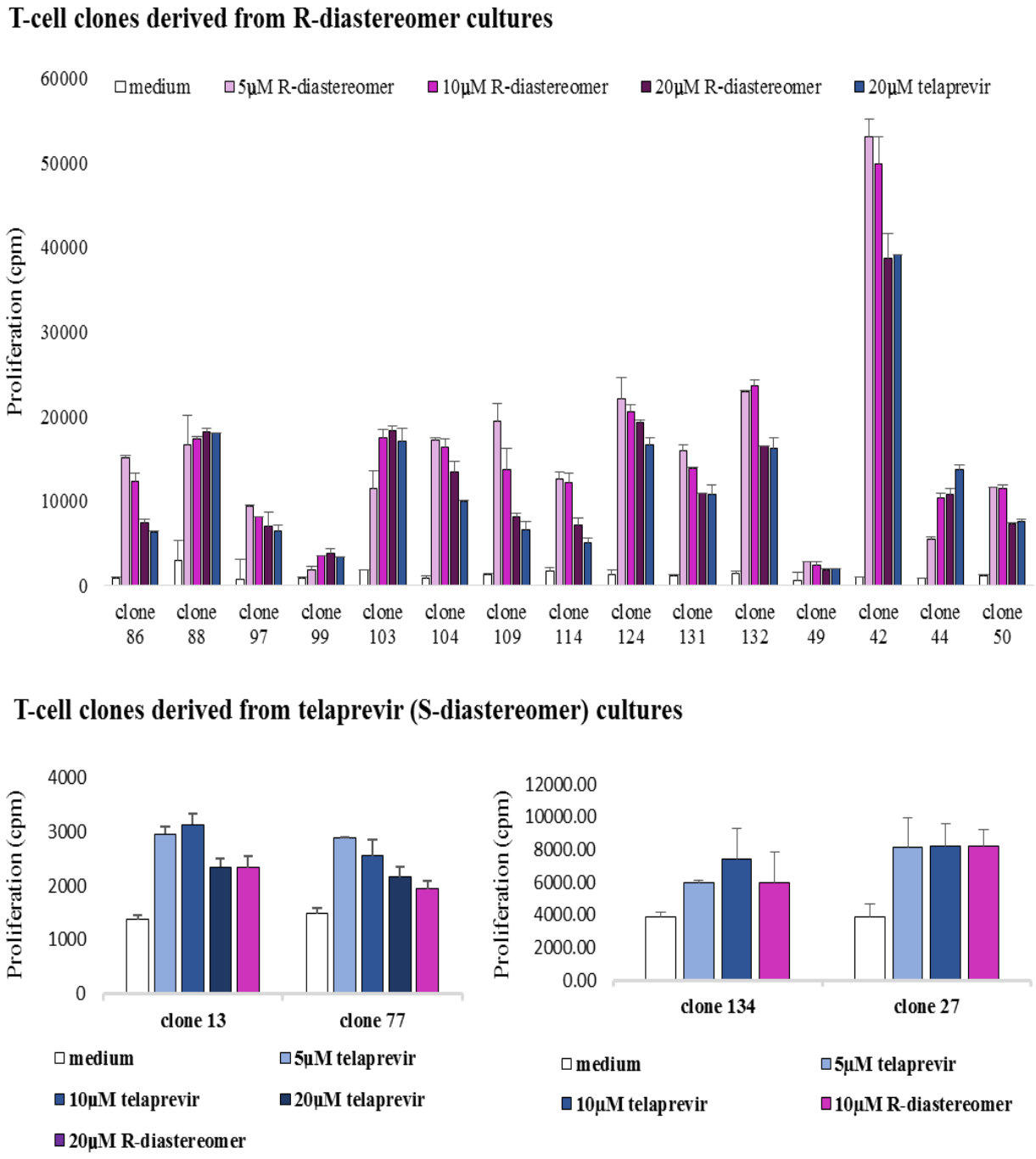


Figure 5-4 Cross-reactivity between telaprevir-diastereomers. Drug-responsive T-cell clones ($5x10^4$/well; 96 well plate; total volume, 200 µL) generated from initial cultures with either the R- or S-diastereomers were cultured (37°C/5% CO2) in triplicate with autologous irradiated EBV-transformed B-cells ($1x10^4$/well) and either diastereomer (5-20 µM) for 48 hrs prior to pulsing with [3H]-thymidine (0.5 µCi/well). After a further 16 hrs incubation, incorporated radioactivity was counted as a measure of proliferation. Data presented as radioactive counts per minute (cpm); error bars indicate the standard deviation for the average of triplicate cultures.

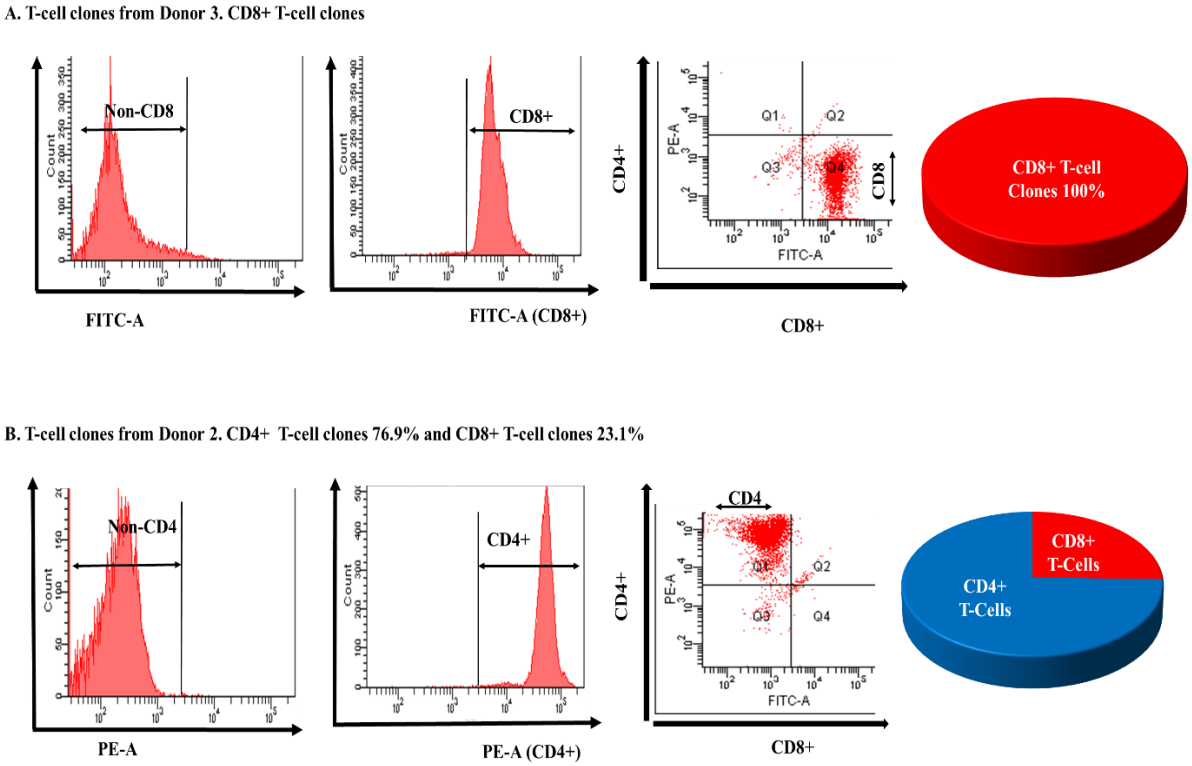


Figure 5-5 Flow cytometry analysis of telaprevir-specific T-cell clones. T-cell clones were generated from 2 healthy donors and stained with CD4 and CD8 fluorochrome antibodies to determine the phenotype. A. shows a dot plot representation of $CD8^+$ telaprevir diastereomer responsive specific T-cell clone phenotypes for donor 3. B. shows a dot plot representation of $CD4^+$ telaprevir specific T-cell clones phenotypes for donor 3.

5.3.3 Telaprevir-responsive T-cells secrete cytotoxic and pro-inflammatory mediators.

ELIspot was utilized to probe for the telaprevir-induced secretion of cytokines and cytolytic molecules from the $CD8^+$ T-cell clones. All clones secreted IFN-γ to a similar degree upon exposure to either telaprevir diastereomer (Figure 5.6). Average spot count across 7 clones shown: IFN-γ; medium, 81 ± 47.9; 10 µM S-diastereomer, 282 ± 77.4; 10 µM R-diastereomer, 288 ± 53). Drug-induced secretion of IL-13 and the cytotoxic mediator granzyme B was similarly observed. In stark comparison, none of the aforementioned mediators were secreted when telaprevir-responsive T-cell clones were exposed to the M11 metabolite (Figure 5.7 A). The lack of cross-reactivity was further confirmed by a negative

proliferative response to the M11 metabolite in these clones (Figure 5.7 B). Secretion of IL-22 was not detected in response to telaprevir diastereomers, despite its reported involvement in inflammatory skin conditions (Figures 5.6 and 5.7).

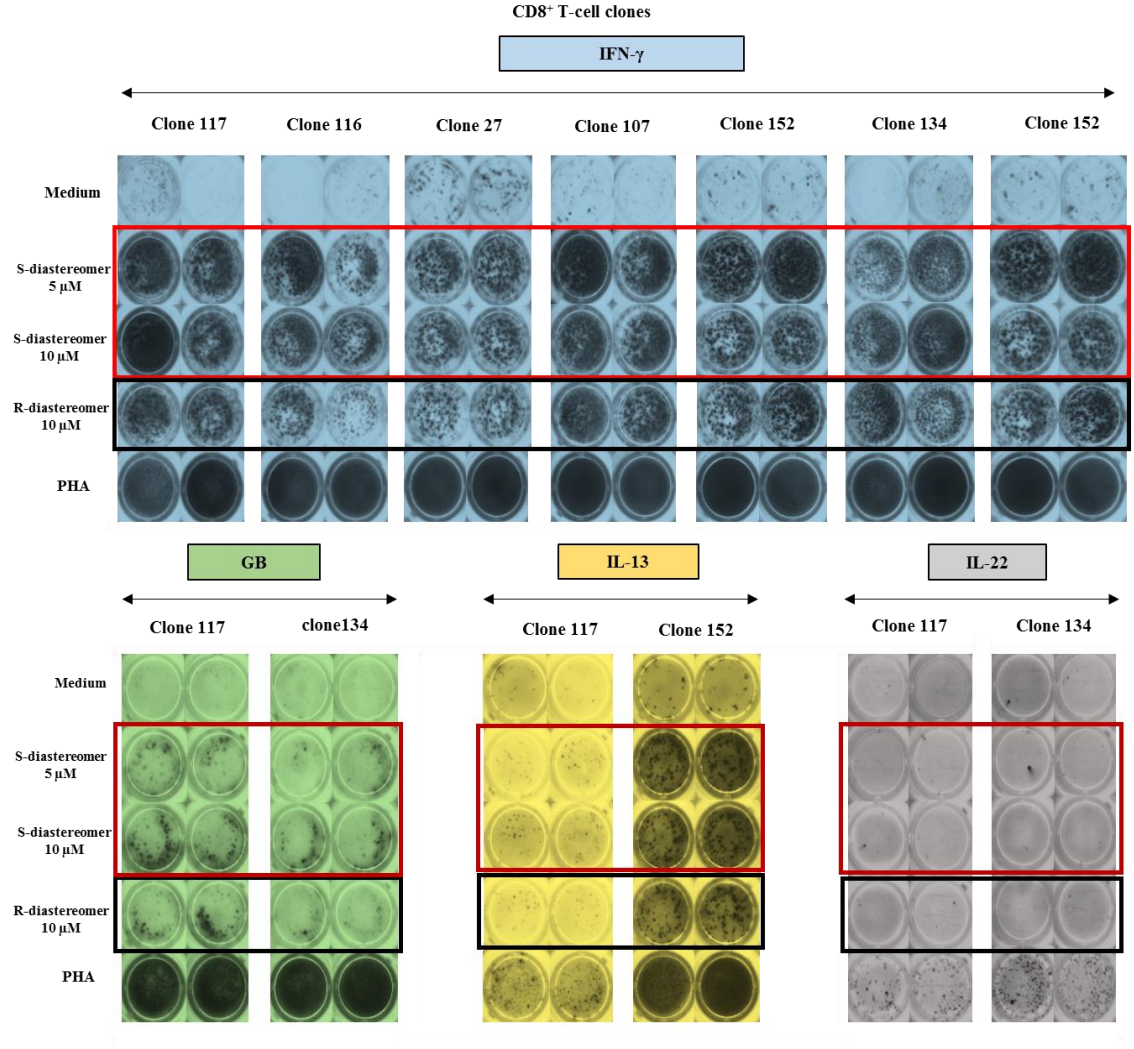


Figure 5-6 Cytokine and cytolytic molecule secretion from telaprevir-responsive T-cell clones. ELIspot plates were coated with IFN-γ, IL-13, IL-22, or granzyme B (GB) capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. T-cell clones ($5x10^4$/well; total volume, 200 µL, 96-well U-bottomed ELIspot plate) were cultured with the S- (5-10 µM) or R-diastereomer of telaprevir and autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After a 48 hrs incubation, the plates were washed and developed in concordance with the manufacturer's instructions. Spot forming units (SFU) counts were analysed from dry wells using an ELIspot reader.* the red box refers to the wells treated with (5-10 µM) S-diastereomer to detect secretion ability for IFN-γ, GB, IL-13, IL-22 respectively.* the black box refers to the wells treated with 10 µM R-diastereomer to detect secretion ability for IFN-γ, GB, IL-13, IL-22 respectively.

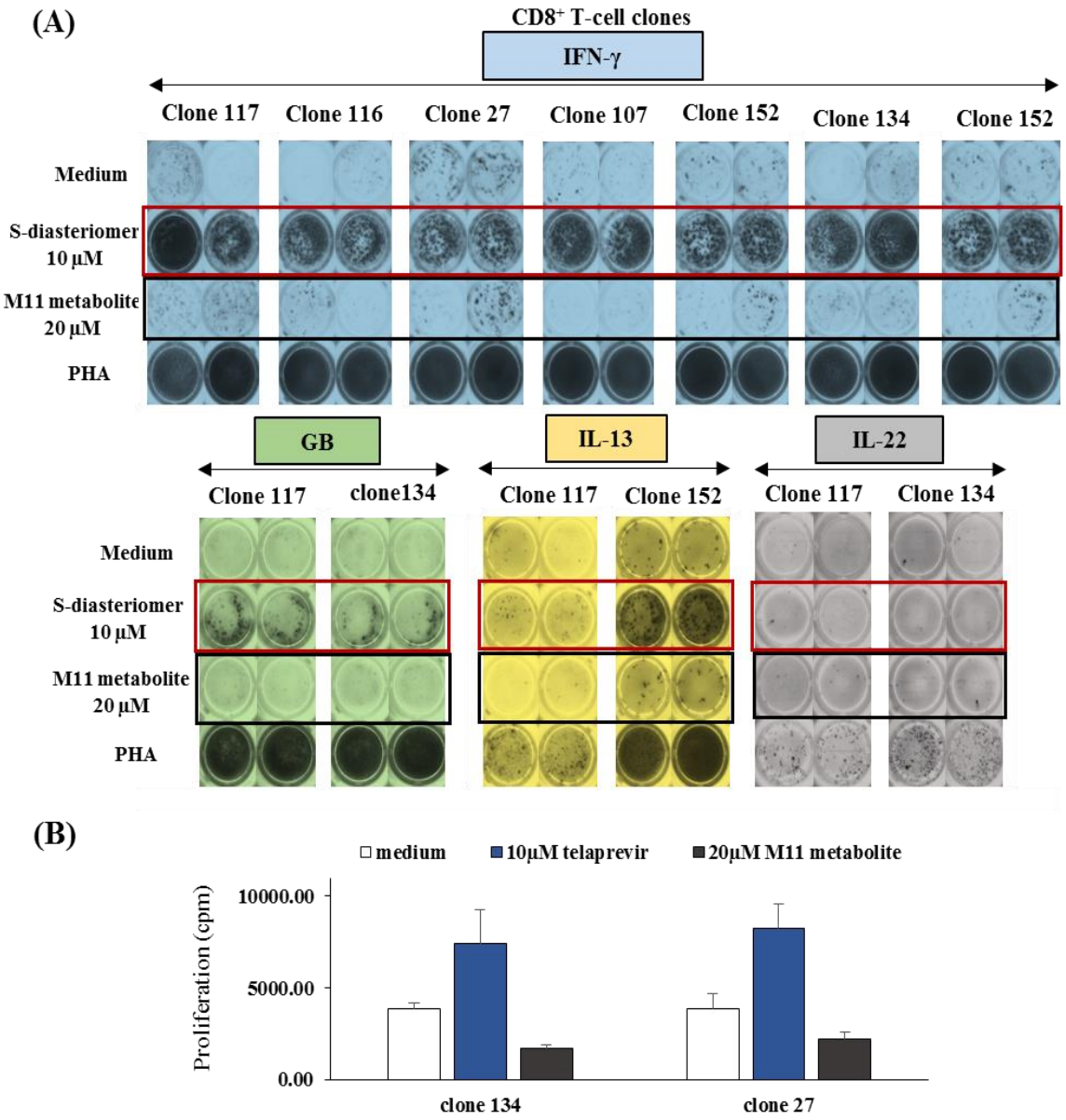


Figure 5-7 The ability of the M11 metabolite to induce (A) cytokine or cytolytic molecule secretion or (B) a proliferative response in telaprevir-responsive T-cell clones. ELIspot plates were coated with IFN-γ, IL-13, IL-22, or granzyme B capture antibody and incubated at 4°C overnight. Wells were then washed and blocked with R9 medium. For both proliferation and ELIspot assays, T-cell clones ($5x10^4$/well; total volume, 200 µL, 96-well plate) were then cultured with telaprevir (10 µM) or the M11 metabolite (20 µM) and autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After a 48 hrs incubation, the ELIspot plates were washed and developed in concordance with the manufacturer's instructions. SFU counts were analysed from dry wells using an ELIspot reader. Alternatively, plates for proliferation analysis were pulsed with [3H] thymidine (0.5 µCi/well) and subject to a further 16 hrs incubation before measurement of incorporated radioactivity. Data presented as radioactive counts per minute (cpm), error bars indicate the standard deviation for the average of triplicate cultures. * The red box refers to the wells treated with (10 µM) S-diastereomer (telaprevir)-specific T-cell clones to detect secretion ability for IFN-γ, GB, IL-13, IL-22 respectively.* the black box refers to the wells treated with (20 µM) M11 metabolite to detect secretion ability for IFN-G, GB, IL-13, IL-22 respectively.

5.3.4 Telaprevir-induced T-cell responses are MHC restricted and occur independent of antigen presenting cell processing.

To assess the presentation of telaprevir to T-cells, telaprevir-specific T-cell clones were first cultured with telaprevir-pulsed autologous irradiated EBV-transformed B-cells free of soluble drug. While T-cells strongly proliferated in response to the soluble drug, they were not stimulated by drug-pulsed antigen presenting cells at either time point (1 or 16 hrs; Figure 5.8 A). Further investigation using HLA blocking antibodies focussed on the requirement for HLA in the activation of T-cells. The telaprevir-induced $CD8^+$ T-cell proliferative response was diminished by blocking HLA class I molecules (Figure 5.8 B), but not the corresponding isotype control. These data indicate that telaprevir is directly presented on HLA class I molecules to passing $CD8^+$ T-cells, without a need for antigen uptake and processing.

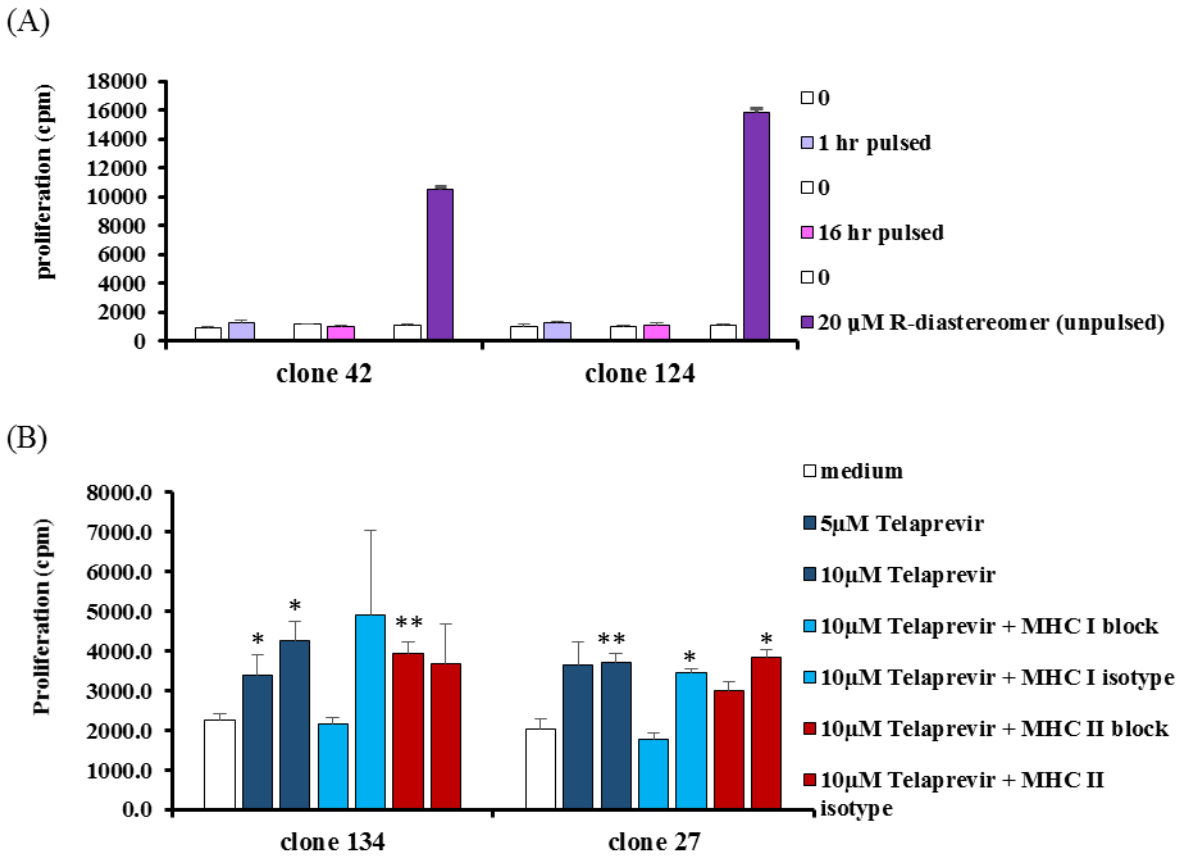
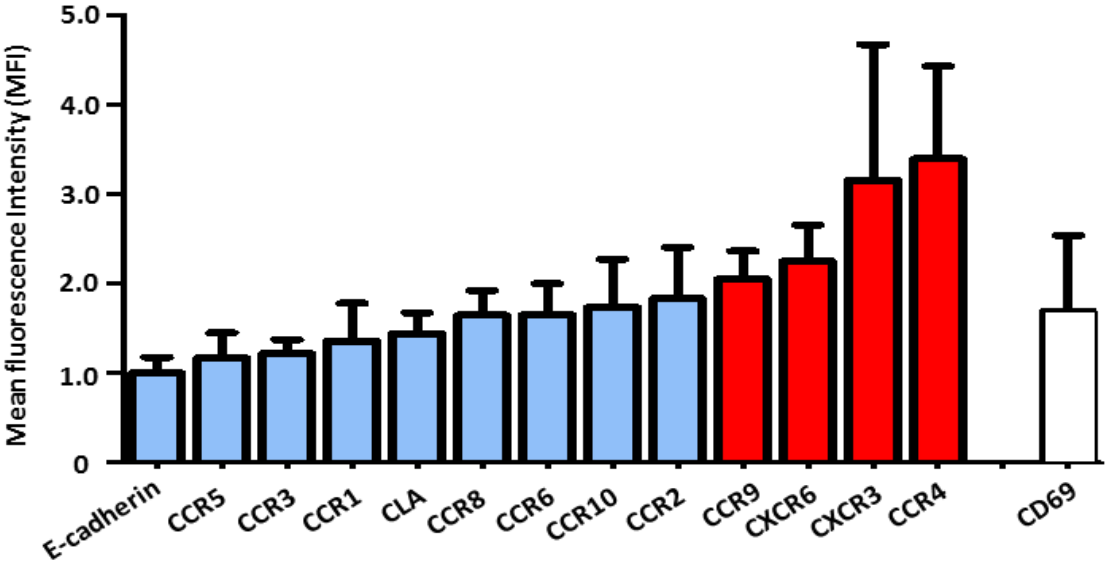


Figure 5-8 Requirement for (A) antigen uptake and (B) HLA alleles for T-cell activation. Autologous EBV were either (A) pulsed with the soluble drug for 1-16 hrs prior to washing to remove free drug, or (B) cultured with HLA blocking antibodies or their respective isotype controls for 30 min. T-cell clones ($5x10^4$/well; total volume, 200 µL, 96-well plate) were then cultured with telaprevir(S-diastereomer) (10-20µM) and pre-conditioned autologous irradiated EBV-transformed B-cells ($1x10^4$/well). After a 48 hrs incubation, cultures were pulsed with [^{3}H]-thymidine (0.5 µCi/well) and subject to a further 16 hrs incubation before measurement of incorporated radioactivity. Data presented as radioactive counts per minute (cpm), error bars indicate the standard deviation for the average of triplicate cultures.

5.3.5 Telaprevir responsive T-cells express distinct TCR Vβ and chemokine receptors.

To induce keratinocyte death, telaprevir-responsive T-cells must express specific homing receptors to promote migration to the skin. The expression of a diverse array of tissue-homing chemokine receptors was analysed on the drug-responsive T-cell clones, which expressed the T-cell activation marker CD69. Of the 13 migratory markers assessed, telaprevir-responsive T-cells most highly expressed CCR4 (skin homing; mean fluorescence intensity [MFI]: 3.40 ± 1.04) and CXCR3 (migration to peripheral tissue; MFI: 3.16 ± 1.52). Further T-cell surface expression analysis revealed a restricted pattern of TCR-Vβ expression, in which there was a high expression of TCR-Vβ 22 (n=6, 46%), with fewer clones expressing TCR-Vβ 2 (n=5, 38%) and TCR-Vβ 5.1 (n=1, 8%). Of note, one clone expressed no identifiable TCR Vβ covered by the kit, which recognises 24 specificities that account for 70% of the total repertoire. Thus, it is likely this T-cell clone expressed another alternative rare TCR-Vβ (Figure 5.9).



CD69	1.7	±	0.8
Chemokines	MFI		
CCR4	3.40	±	1.04
CXCR3	3.16	±	1.52
CXCR6	2.25	±	0.41
CCR9	2.06	±	0.31
CCR2	1.83	±	0.57
CCR10	1.74	±	0.54
CCR6	1.66	±	0.35
CCR8	1.65	±	0.28
CLA	1.44	±	0.23
CCR1	1.36	±	0.42
CCR3	1.22	±	0.15
CCR5	1.17	±	0.28
E CAD	1.01	±	0.17

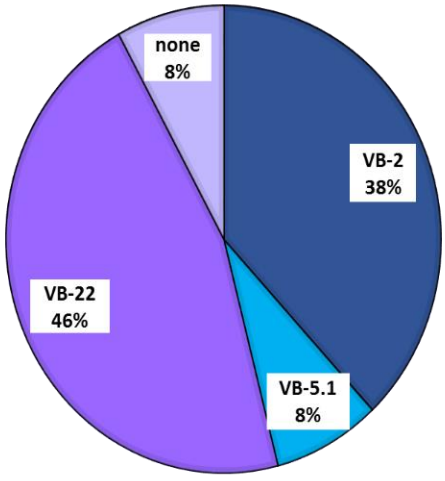


Figure 5-9 TCR Vβ and chemokine profile of telaprevir-responsive T-cell clones. T-cell clones ($5x10^4$) were washed and stained with (A) individual Fluorochrome-conjugated antibodies specific for chemokine receptors or (B) combined Fluorochrome-conjugated antibodies specific for TCR Vβ specificities. Samples were left in the dark for 20 min at 4°C to aid antibody binding. After which samples were washed to remove unbound antibody and fixed in 4% PFA prior to analysis using a BD FACS CANTO II flow cytometer. Data analysis was performed using cyflogic software.

5.4 Discussion.

As the acute stage of HCV is largely asymptomatic, the majority of patients develop a chronic infection that leads to long term, life threatening liver complications. An adaptation of the standardised dual therapy, by co-administration of telaprevir, led to >30% increase in therapeutic response rate. However, telaprevir also enhanced the incidence of mild skin reactions and led to a number of patients developing life threatening hypersensitivity reactions including DRESS and SJS. The appearance of new drugs with improved safety profile resulted in telaprevir being withdrawn from the market.

Drug-specific T-cells have been isolated from the blood and blister fluid of patients with other forms of severe hypersensitivity reaction, and these T-cells are thought to mediate tissue destruction. However, as yet telaprevir-responsive T-cells have not been identified. While samples from hypersensitive patients are informative for clinical diagnosis (as detected in chapter 3 with dapsone), they represent a memory T-cell response and give no indication regarding the ability of the antigen to activate the more highly regulated naïve population. Thus, using PBMC drug bulk cultures we probed the immunogenicity of the telaprevir S- and R-diastereomers.

Telaprevir-responsive T-cell clones were generated from 3 out of 7 healthy volunteers; however, the frequency of identifiable drug-responsive T-cell clones was low. Although administered as the S-diastereomer, telaprevir spontaneously converts to the R-diastereomer *in vivo*. A study on Japanese HCV patients showed that the mean C_{max} for telaprevir was 5.4 µM at steady state (Yamada et al., 2012), where maximal plasma concentrations of the R-diastereomer were almost equivalent to those of the S-diastereomer (Nakada et al., 2014). In our study, T-cell clones were derived from PBMC cultured with both the S- and R-diastereomer. In contrast to the diastereomer-specific actions of telaprevir at its pharmacological target, the hepatitis C viral enzyme NS3/4A serine protease, a high degree

of T-cell cross reactivity was observed with both diastereomers. In fact, the same concentrations of S- and R-diastereomer were capable of inducing proliferate responses to a similar degree. While these data imply the parent compound is responsible for T-cell activation, the formation of a metabolite after internalisation by antigen presenting cells and subsequent presentation on HLA to passing T-cells is a possibility. One potentially immunogenic metabolite is M11, as it has previously been reported to induce a positive T-cell response in experimental animals. Telaprevir-responsive T-cell clones failed to respond to M11, and moreover, cloning directly using the M11 metabolite was unsuccessful in 3 donors, including the donors who provided the most clones responsive towards the parent compound. While this inter-species differential response may relate to the small number of animals or humans tested between the two studies, it nonetheless stresses the importance of assessing antigenicity and immunogenicity using human models.

Telaprevir is a reversible covalent binding inhibitor of its pharmacological target. The drug interacts with a high degree of specificity with serine in position 139, which resides in the catalytic area of the protease enzyme. Dissociation of the covalently bound drug has a half-life of 58 min (Fowell and Nash, 2010a).

To investigate whether the telaprevir-responsive T-cells were activated through the formation of protein adducts by a hapten mechanism or through direct HLA binding, antigen presenting cell pulsing experiments were conducted. Haptenic drugs bind irreversibly to antigen presenting cells in the pulsing assay and stimulate T-cells after repeated washing to remove the non-covalently-bound drug. In contrast, drugs that bind directly to HLA through a reversible bond yield a negative result (Alzahrani et al., 2017b, Castrejon et al., 2010a, Schnyder et al., 2000). While the telaprevir-specific T-cell response was MHC restricted, the T-cells were only activated in the presence of the soluble drug, highlighting that formation of a covalent adduct, antigen-uptake and processing by antigen presenting cells is not a

requirement for T-cell activation. These data indicate that telaprevir may be able to activate T-cells expressing distinct TCR Vβs via a direct HLA binding interaction. Interestingly, telaprevir is relatively large, with a molecular weight of 680 Da. This equates to the over 50% of the mass of a typical HLA class I binding peptide (assuming an average amino acid mass of 130 Da and 9-10 amino acids in the peptide sequence). Thus, it will be intriguing to discover the structure of the telaprevir HLA peptide binding interaction. It seems unlikely that telaprevir will fit into a binding pocket under an HLA binding peptide. The only other possibilities are that telaprevir (1) interacts with the HLA binding peptide and projects from the HLA molecule, (2) replaces the requirement for an HLA binding peptide and (3) binds elsewhere on the HLA molecule altering the structure of the HLA binding cleft.

$CD8^+$ T-cells are designed to inflict damage and thus are the most likely mediators of keratinocyte death in patients with telaprevir hypersensitivity. Thus, $CD8^+$ T-cells were analysed for their cytokine secretion profile and the secretion of cytolytic molecules using ELIspot. Previously, Suda *et al* reported a correlation between the level of the cytotoxic mediator granulysin and the severity of telaprevir-induced skin reactions. Moreover, they describe an early rise in serum granulysin levels with the onset of severe symptoms which fades within 6 days and therefore concluded that granulysin can be utilized as an early predictive marker for telaprevir-induced skin reactions (Suda et al., 2015). In agreement with the induction of a cytotoxic response, telaprevir-responsive $CD8^+$ T-cell clones not only secreted IFN-γ and IL-13, but also the cytotoxic mediator granzyme B in response to culture with either diastereomer. Upon release from cytotoxic T-cell granules, granzyme B enters target tissue to cleave caspases and initiate apoptosis. The secretion of IL-22 was additionally monitored due to its proposed role in inflammatory skin conditions, including psoriasis. Furthermore, IL-22-secreting cells have been identified in patients with allergic contact dermatitis and β-lactam hypersensitivity reactions (Akdis et al., 2012, Cavani et al., 2012,

Eyerich et al., 2010, Sullivan et al., 2018). IL-22 was not secreted by telaprevir-responsive T-cells in this study.

Chemokine receptor pattern analysis on telaprevir-responsive T-cell clones detected a population with a skin-homing phenotype. While the skin homing receptor CCR10 was present, CCR4 and CXCR3 were also highly expressed. CXCR3 is predominantly expressed by Th1 T-cells while CCR4 is expressed by Th2 T-cells (Kim et al., 2001a). These findings, along with the antigen-specific secretion of IFN-γ (Th1) and IL-13 (Th2), suggest the presence of a mixed Th1/2 T-cell population. CCR9, characterised as a gut homing receptor for T-cells with a role in intestinal inflammation (Agace, 2008, Bekker et al., 2015), was also relatively highly expressed in comparison to other chemokine receptors. Interestingly, adverse intestinal effects with telaprevir hypersensitivity is very common ranging from diarrhoea to haemorrhoids in 25% and 12% of patients, respectively. As yet, the underlying mechanism of intestinal disruption is not defined, but this data indicates the migration of T-cells may play a role.

This study identifies and characterises the telaprevir-induced activation of T-cells from 3 out of 7 healthy drug-naïve donors that develop into skin-homing, cytotoxic T-cells, which are activated by an HLA-restricted, but processing-independent mechanism. Drug-specific T-cells responded to either telaprevir diastereomer and expressed varied TCRs. Drug-responsive T-cells were induced using cultures from drug-naïve healthy donors and so define the utility of *in vitro* human platforms to explore the requirements for T-cell activation. As studies have previously failed to associate telaprevir hypersensitivity with HLA risk alleles (Roujeau et al., 2013b), the development of *in vitro* human assays to allow (a) modulation of other immune parameters and (b) inclusion of autologous keratinocytes will be key to understand the inter-individual skin-specific targeting by the immune system

Chapter 6: General Discussion.

1.6 General Discussion.

Drugs are critical for the treatment and prevention of diseases in modern medicine, but they can also cause significant unwanted adverse drug reactions. Adverse drug reactions contribute toward increased hospitalization and are a major burden on health care services throughout the world. Many reactions are self-limiting through drug withdrawal or a dose reduction. However, a small number of adverse drug reactions develop into life-threatening conditions. The majority of these are thought to involve the adaptive immune system; however, the mechanisms of drug-specific immune cell activation and factors that determine individual susceptibility are not well defined. Since the disease pathogenesis is not defined, it has not been possible to generate sensitive and specific assays for use by industry to predict intrinsic immunogenicity of new chemical entities or the National Health Service to diagnose culprit drugs in patients with reactions.

Adverse reactions that involve the drug-specific activation of the adaptive immune system are often referred to as hypersensitivity or allergy. The onset of clinical symptoms may involve the production of antibodies by B-cells; however, the most severe and most unpredictable involve T-cells. Many forms of T-cell-mediated drug hypersensitivity reaction targeting skin, liver and blood have been shown to develop more frequently in individuals expressing a single HLA class I or class II allele (Daly and Day, 2012, Pirmohamed et al., 2015, Redwood et al., 2018). These data suggest that the protein encoded by the HLA allele is directly involved in presenting the drug antigen to the T-cells that bring about the clinical symptoms of the hypersensitivity reaction. Drugs can interact directly with HLA alleles via a reversible pharmacological interaction to promote T-cell activation (Pichler et al., 2011). The drug abacavir interacts with HLA B*57:01 deep within the peptide binding cleft (Illing et al., 2012, Ostrov et al., 2012). Abacavir binding alters the peptide repertoire displayed on the surface of antigen presenting cells by HLA-B*57:01. It has been proposed that these

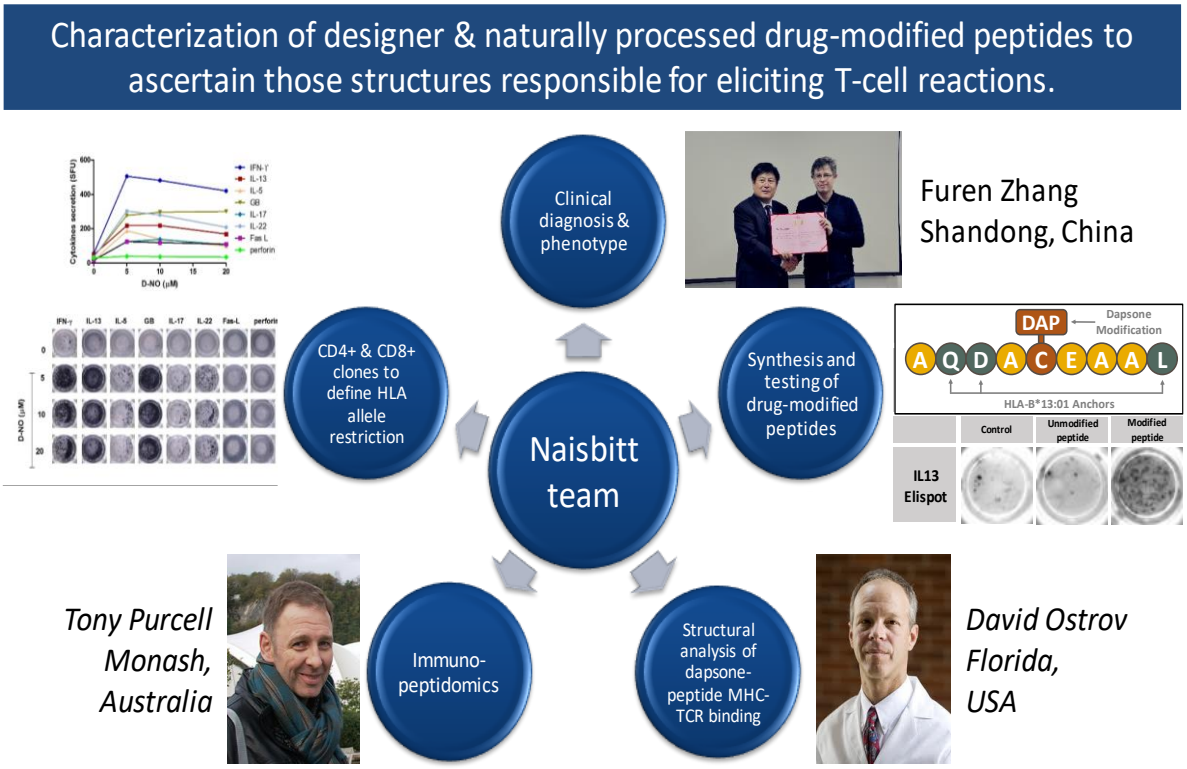
altered peptide sequences are responsible for $CD8^+$ T-cell activation in patients with abacavir hypersensitivity; however, the nature of the peptides that stimulate the T-cell response are yet to be defined. For other drug reactions associated with expression of a specific HLA allele, the nature of the drug HLA binding interaction is less well defined. Drugs such as allopurinol and carbamazepine are thought to bind directly to the linked HLA molecules (Yun et al., 2014, Wei et al., 2012). The binding could take place in the peptide binding cleft similar to abacavir; however, it is also feasible that the drugs interact with HLA bound peptides above the binding cleft or the HLA molecule itself at a site distal to the peptide binding interaction. Other drugs such as flucloxacillin and amoxicillin do not interact directly with HLA molecules expressed by patients with drug-induced liver injury (Monshi et al., 2013b, Yaseen et al., 2015, Kim et al., 2015). Instead, the drugs bind covalently to non-HLA associated protein generating neo-epitopes. These modified proteins are naturally processed by antigen presenting cells. This liberates HLA binding peptides that contain the drug moiety bound to specific amino acid residues. Although the nature of the naturally eluted drug-modified peptides that activate T-cells has not been defined, synthesis of designer drug modified peptides containing amino acids that interact with specific HLA molecules has shown that patient T-cells display specificity for the peptide sequence and the drug molecule bound to specific locations in the peptide sequence (Padovan et al., 1996). Importantly, to date, studies have not been conducted to define the binding interaction of reactive drug metabolites with HLA molecules linked to specific forms of drug hypersensitivity. Thus, the aim of this thesis was to focus on three forms of drug hypersensitivity to explore (1) the role of drug-specific T-cells and (2) the nature of the drug HLA binding interaction that leads to T-cell activation. Telaprevir and tolvaptan have recently been associated with skin and liver reactions with a delayed onset (Fowell and Nash, 2010b, Watkins et al., 2015b). Specific HLA alleles are not linked to telaprevir reactions (Roujeau et al., 2013b), whereas genetic

studies to explore the association between expression of HLA alleles and development of tolvaptan-induced liver injury have not been performed. Furthermore, with both reactions, the role of the adaptive immune system in the disease pathogenesis has not been defined. Dapsone is an older drug that has been associated with severe skin and liver reactions for many years. Recently the development of dapsone-induced DRESS in patients of Chinese ancestry was found to be strongly associated with expression of the class I HLA allele B*13:01 (Zhang et al., 2013). Dapsone is metabolized to a hydroxylamine intermediate that undergoes spontaneous oxidation to generate nitroso dapsone. Nitroso dapsone is highly reactive and binds covalently to cysteine residues on proteins (Vyas et al., 2006b, Vyas et al., 2006c). Before arriving for my PhD studies, researchers in Liverpool synthesized nitroso dapsone in a stable form (Alzahrani et al., 2017a). This allowed an assessment of the immunogenicity of dapsone and nitroso dapsone in patients that developed DRESS.

For the dapsone project we collaborated with the authors of the original genetic association study to recruit HLA-B*13:01+ patients with dapsone-mediated DRESS syndrome. Patient PBMC were activated with the parent drug and the nitroso metabolite. These data provided the framework to clone T-cells to characterize the cellular pathophysiology of the reaction, to explore the nature of the antigen that activated T-cells and to define crossreactivity with the parent drug, metabolites and closely related structures. Dapsone and nitroso dapsone activated $CD4^+$ and $CD8^+$ T-cells that secreted a similar panel of cytokines. Three patterns of reactivity were detected with dapsone- and nitroso dapsone-responsive clones. Certain clones were highly specific and were only activated with one compound the drug or metabolite. Others displayed high and low levels of cross reactivity. Activation of the clones with the parent drug occurred in an HLA-restricted manner through a direct binding of the drug with HLA and the T-cell receptor. In contrast, clones displaying reactivity against the

nitroso metabolite were activated via a hapten mechanism involving the formation of a drug protein adduct.

Through the generation of a panel of antigen presenting cells expressing defined, but differing HLA B alleles we demonstrated that the drug and drug metabolite antigen could interact selectively with HLA-B*13:01, the HLA allele identified in the genetic association studies, to stimulate certain $CD8^+$ T-cell clones. These data provide the framework for future studies to identify the nature of the drug and drug metabolite binding interaction with HLA-B*13:01. In ongoing experiments, designer HLA-B*13:01 binding peptides containing modifiable cysteine residues are being synthesized and cultured with nitroso dapsone to generate adducts. These will be then assessed for their ability to activate patient T-cells. Alongside these experiments, peptides naturally eluted from dapsone- and nitroso dapsone-treated antigen presenting cells are being assessed by mass spectrometry to identify drug(metabolite)-induced changes in the immunopeptidome and the nature of drug-modified HLA B*13:01 binding peptides. We are working alongside collaborators in China, America and Australia to attempt to solve the X-ray crystal structure of nitroso dapsone-modified peptides associated with HLA-B*13:01 and specific T-cell receptors. Scheme 6.1 shows the study plan and the team of investigators assembled to meet the study objectives.



To recruit patients with tolvaptan-induced liver injury, we collaborated with Otsuka Pharmaceuticals, who market the drug. Patients from Asia, America and Europe were identified and recruited to the study. Blood samples were isolated and viable PBMC cryopreserved at various clinical centres for Liverpool to explore whether tolvaptan or the major metabolites of tolvaptan activate T-cells. In contrast to the patients with dapsone-induced DRESS, PBMC from patients with tolvaptan-induced liver injury were not stimulated to proliferate with tolvaptan or its metabolite. Despite this, cloning experiments were conducted by serial dilution of drug-treated PBMC and repetitive mitogen-driven expansion. On initial testing for drug- or drug metabolite-specific proliferation, a small number of responsive clones were detected. These clones were expanded further so that each clone could be assessed for proliferative responses and cytokine secretion after drug stimulation. The majority of clones yielded negative results in both proliferation and cytokine release assays on repeated testing. However, a small number of clones displayed

concentration-dependent responses, mainly in the presence of the tolvaptan metabolite DM-4107. It was difficult to expand these clones in great numbers, thus, it was impossible to perform a detailed analysis of cellular phenotype or function. Thus, in a new project initiated after completion of the patient study, large numbers of tolvaptan antigen-responsive T-cell clones have been generated from drug-naïve healthy donors after regulatory T-cells were removed. The vast majority of these clones were responsive to DM-4107 and not the parent drug. The reason why it was so difficult to clone T-cells from patients with tolvaptan-induced liver injury is not clear. It may be that the frequency of drug-specific T-cells that circulate in the periphery of patients is very low. However, it could also relate the fact that the PBMC were isolated and cryopreserved in several different countries. Although cells from most patients retained adequate viability, it is possible that they were functionally impaired.

Telaprevir, a drug developed for use in combination with pegylated interferon and ribavirin to treat Hepatitis C, was the final study drug. Telaprevir increases the incidence of cutaneous reactions seen in patients when compared with pegylated interferon and ribavirin treatment alone. Moreover, several cases of Stevens Johnson syndrome were observed in patients receiving the triple therapy. The appearance of the severe cutaneous reactions and the development of newer equally efficacious drugs eventually lead to the withdrawal of telaprevir. As patients with telaprevir-induced skin reactions were not readily available, PBMC from healthy drug-naïve donors were used to explore the telaprevir immunogenicity. This was possible as telaprevir reactions are not associated with expression of a specific HLA allele (Roujeau et al., 2013b). T-cells cloned from the peripheral blood of certain donors displayed reactivity against telaprevir. As telaprevir exists in different isomeric forms, with only one form showing pharmacological activity, PBMC were cultured with both isomers of the drug to generate clones. Clones displaying reactivity against both isomers were detected and importantly all responsive clones were stimulated to proliferate and

secrete cytokines to a similar extent with the same concentrations of the isomers. Mechanistic studies revealed that telaprevir and its alternative isomer interacts directly with HLA molecules to stimulate the clones. These data highlight an important difference in the binding interaction of telaprevir with its desired pharmacological and unwanted immunological target. If PBMC from patients were available it would be interesting to see whether our findings are replicated and whether the phenotype of drugs-specific T-cells are the same or different.

To conclude, the data presented in this thesis shows that drug-specific T-cells participate in hypersensitivity reactions that target skin and liver. Furthermore, it is possible to utilize PBMC from healthy donors to model the T-cell responses that develop in patients. In the long term these mechanistic studies should feed into attempts to develop predictive T-cells assays for industry to predict immunogenicity of new chemical entities and diagnostic T-cell assays to improve management of hypersensitive patients.

Chapter 7: Bibliography.

Abbas, A. K., Lichtman, A. H. and Pillai, S. (2017) *Cellular and Molecular Immunology E-Book.* Elsevier.

Abbas, A. K., Lichtman, A. H., Pillai, S., Baker, D. L. and Baker, A. (2018) *Cellular and molecular immunology.*

Abul K Abbas, A. H. L., Shivi Pillai. (2015) *Cellular and molecular IMMUNOLOGY.* Eight edition edn.

Actor, J. K. (2014) 'Chapter 4 - T Lymphocytes: Ringleaders of Adaptive Immune Function', *Introductory Immunology*. Amsterdam: Academic Press, pp. 42-58.

Adam, J., Pichler, W. J. and Yerly, D. (2011) 'Delayed drug hypersensitivity: models of T-cell stimulation', *Br J Clin Pharmacol,* 71(5), pp. 701-7.

Agace, W. W. (2008) 'T-cell recruitment to the intestinal mucosa', *Trends in Immunology,* 29(11), pp. 514-522.

Aivazian, D. and Stern, L. J. (2000) 'Phosphorylation of T cell receptor zeta is regulated by a lipid dependent folding transition', *Nat Struct Biol,* 7(11), pp. 1023-6.

Akdis, M., Palomares, O., van de Veen, W., van Splunter, M. and Akdis, C. A. (2012) 'TH17 and TH22 cells: a confusion of antimicrobial response with tissue inflammation versus protection', *The Journal Of Allergy And Clinical Immunology,* 129(6), pp. 1438.

Alberts, B. (2002) *Molecular biology of the cell. [electronic book]. Free online access: Pubmed*: New York : Garland Science, c2002.

4th ed.

Alberts, B., Johnson, A., Lewis, J. and .2002., e. a. (2002) 'Molecular Biology of the Cell. Chapter 24, The Adaptive Immune System. '. 4th edition ed. New York: Garland Science.

Alegre, M. L., Leemans, J., Le Moine, A., Florquin, S., De Wilde, V., Chong, A. and Goldman, M. (2008) 'The Multiple Facets of Toll-Like Receptors in Transplantation Biology', (no. 1), pp. 1.

Alfirevic, A., Alsbou, M., Park, B. K., Pirmohamed, M., Vilar, F. J., Jawaid, A., Thomson, W., Ollier, W. E. R., Bowman, C. E. and Delrieu, O. (2009) 'TNF, LTA, HSPA1L and HLA-DR gene polymorphisms in HIV-positive patients with hypersensitivity to cotrimoxazole', *Pharmacogenomics,* 10(4), pp. 531-540.

Alfirevic, A., Gonzalez-Galarza, F., Bell, C., Martinsson, K., Platt, V., Bretland, G., Evely, J., Lichtenfels, M., Cederbrant, K., French, N., Naisbitt, D., Park, B. K., Jones, A. R. and Pirmohamed, M. (2012) 'In silico analysis of HLA associations with drug-induced liver injury: use of a HLA-genotyped DNA archive from healthy volunteers', *Genome Medicine,* 4(6), pp. 51-51.

Allen, C. D., Okada, T. and Cyster, J. G. (2007) 'Germinal-center organization and cellular dynamics', *Immunity*, 27(2), pp. 190-202.

Alzahrani, A., Ogese, M., Meng, X., Waddington, J. C., Tailor, A., Farrell, J., Maggs, J. L., Betts, C., Park, B. K. and Naisbitt, D. (2017a) 'Dapsone and Nitroso Dapsone Activation of Naive T-Cells from Healthy Donors', *Chem Res Toxicol,* 30(12), pp. 2174-2186.

Alzahrani, A., Ogese, M., Meng, X., Waddington, J. C., Tailor, A., Farrell, J., Maggs, J. L., Betts, C., Park, B. K. and Naisbitt, D. (2017b) 'Dapsone and Nitroso Dapsone Activation of Naive T-Cells from Healthy Donors', *Chem Res Toxicol*.

Andres, E., Zimmer, J., Affenberger, S., Federici, L., Alt, M. and Maloisel, F. (2006) 'Idiosyncratic drug-induced agranulocytosis: Update of an old disorder', *Eur J Intern Med,* 17(8), pp. 529-35.

Andrews, E., Armstrong, M., Tugwood, J., Swan, D., Glaves, P., Pirmohamed, M., Aithal, G. P., Wright, M. C., Day, C. P. and Daly, A. K. (2010) 'A role for the pregnane X receptor in flucloxacillin-induced liver injury', *Hepatology,* 51(5), pp. 1656-64.

Ang, A. Y., Palmon, F. E. and Holland, E. J. (2013) '30 - Erythema Multiforme, Stevens–Johnson Syndrome and Toxic Epidermal Necrolysis', *Ocular Surface Disease: Cornea, Conjunctiva and Tear Film*. London: W.B. Saunders, pp. 231-241.

Arango Duque, G. and Descoteaux, A. (2014) 'Macrophage cytokines: involvement in immunity and infectious diseases', *Frontiers in immunology,* 5, pp. 491-491.

Ariza, A., Mayorga, C., Fernandez, T. D., Barbero, N., Martin-Serrano, A., Perez-Sala, D., Sanchez-Gomez, F. J., Blanca, M., Torres, M. J. and Montanez, M. I. (2015) 'Hypersensitivity reactions to beta-lactams: relevance of hapten-protein conjugates', *J Investig Allergol Clin Immunol,* 25(1), pp. 12-25.

Aronson, J. K. (2012) 'Adverse Drug Reactions: History, Terminology, Classification, Causality, Frequency, Preventability', *Stephens' Detection and Evaluation of Adverse Drug Reactions*.

Artyomov, M. N., Lis, M., Devadas, S., Davis, M. M. and Chakraborty, A. K. (2010) 'CD4 and CD8 binding to MHC molecules primarily acts to enhance Lck delivery', *Proceedings of the National Academy of Sciences,* 107(39), pp. 16916.

Asscher, A. W., Parr, G. D. and Whitmarsh, V. B. (1995) 'Towards the safer use of medicines', *Bmj,* 311(7011), pp. 1003-6.

Aster, R. H. (2010) 'Adverse drug reactions affecting blood cells', *Handb Exp Pharmacol,* (196), pp. 57-76.

Bachmann, M. F., Kopf, M. and Marsland, B. J. (2006) 'Chemokines: more than just road signs', *Nature Reviews. Immunology,* 6(2), pp. 159-164.

Bain, B. J. (2017) 'Structure and function of red and white blood cells', *Medicine,* 45(4), pp. 187-193.

Baldo, B. A. and Pham, N. H. (2013) *Drug allergy. [electronic book] : clinical aspects, diagnosis, mechanisms, structure-activity relationships. Online access with purchase: Springer*: New York, NY : Springer, 2013.

Bassing, C. H., Swat, W. and Alt, F. W. (2002) 'The mechanism and regulation of chromosomal V(D)J recombination', *Cell,* 109 Suppl, pp. S45-55.

Baumgarth, N. (2003) 'A two-phase model of B-cell activation', *Immunological Reviews,* 176(1), pp. 171-180.

Baumjohann, D., Preite, S., Reboldi, A., Ronchi, F., Ansel, K. M., Lanzavecchia, A. and Sallusto, F. (2013) 'Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype', *Immunity,* 38(3), pp. 596-605.

Beenhouwer, D. O. (2018) 'Chapter 17 - Molecular Basis of Diseases of Immunity', in Coleman, W.B. & Tsongalis, G.J. (eds.) *Molecular Pathology (Second Edition)*: Academic Press, pp. 329-345.

Bekker, P., Ebsworth, K., Walters, M. J., Berahovich, R. D., Ertl, L. S., Charvat, T. T., Punna, S., Powers, J. P., Campbell, J. J., Sullivan, T. J., Jaen, J. C. and Schall, T. J. (2015) 'CCR9 Antagonists in the Treatment of Ulcerative Colitis', *Mediators Inflamm,* 2015, pp. 628340.

Bell, C. C., Faulkner, L., Martinsson, K., Farrell, J., Alfirevic, A., Tugwood, J., Pirmohamed, M., Naisbitt, D. J. and Park, B. K. (2013) 'T-Cells from HLA-B*57:01+ Human Subjects Are Activated with Abacavir through Two Independent Pathways and

Induce Cell Death by Multiple Mechanisms', *Chemical Research in Toxicology*, 26(5), pp. 759-766.

Benesic, A., Rotter, I., Dragoi, D., Weber, S., Buchholtz, M.-L. and Gerbes, A. L. (2018) 'Development and Validation of a Test to Identify Drugs That Cause Idiosyncratic Drug-Induced Liver Injury', *Clinical Gastroenterology and Hepatology*.

Bennett, P. N., Brown, M. J. and Sharma, P. (2012) *Clinical pharmacology. [electronic book]. Online access with purchase: Dawsonera (Annual limit multiple access)*: Oxford : Churchill Livingstone, 2012.

11th ed.

Berard, M. and Tough, D. F. (2002) 'Qualitative differences between naïve and memory T cells', *Immunology*, 106(2), pp. 127-138.

Bergström, M. A., Ott, H., Carlsson, A., Neis, M., Zwadlo-Klarwasser, G., Jonsson, C. A. M., Merk, H. F., Karlberg, A. T. and Baron, J. M. (2007) 'A skin-like cytochrome P450 cocktail activates prohaptens to contact allergenic metabolites', *Journal of Investigative Dermatology*, 127(5), pp. 1145-1153.

Bettelli, E., Korn, T. and Kuchroo, V. K. (2007) 'Th17: the third member of the effector T cell trilogy', *Curr Opin Immunol*, 19(6), pp. 652-7.

Bettini, M. L., Chou, P.-C., Guy, C. S., Lee, T., Vignali, K. M. and Vignali, D. A. A. (2017) 'Cutting Edge: CD3 ITAM Diversity Is Required for Optimal TCR Signaling and Thymocyte Development', *The Journal of Immunology*, 199(5), pp. 1555.

Betts, R. J. and Kemeny, D. M. (2009) 'CD8+ T cells in asthma: friend or foe?', *Pharmacol Ther*, 121(2), pp. 123-31.

Bharadwaj, M., Illing, P., Theodossis, A., Purcell, A. W., Rossjohn, J. and McCluskey, J. (2012) 'Drug hypersensitivity and human leukocyte antigens of the major histocompatibility complex', *Annu Rev Pharmacol Toxicol*, 52, pp. 401-31.

Bix, M., Liao, N. S., Zijlstra, M., Loring, J., Jaenisch, R. and Raulet, D. (1991) 'Rejection of class I MHC-deficient haemopoietic cells by irradiated MHC-matched mice', *Nature*, 349(6307), pp. 329-31.

Blair, H. A. and Keating, G. M. (2015) 'Tolvaptan: A Review in Autosomal Dominant Polycystic Kidney Disease', *Drugs*, 75(15), pp. 1797-806.

Bloom, A. K. and Ryan, E. T. (2013) 'Dapsone', *Hunter's Tropical Medicine and Emerging Infectious Disease (Ninth Edition)*. London: W.B. Saunders, pp. 1108-1109.

Boegel, S. E., Bodis, G., Toth, V., Schwarting, A. and Walker, J. M. S. E. 2018. Role of Human Leukocyte Antigens (HLA) in Autoimmune Diseases. New York, NY: Humana Press.

Boehm, T. and Rabbitts, T. H. (1989) 'The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors', *Faseb j*, 3(12), pp. 2344-59.

Bouvy, J. C., De Bruin, M. L. and Koopmanschap, M. A. (2015) 'Epidemiology of adverse drug reactions in Europe: a review of recent observational studies', *Drug safety*, 38(5), pp. 437-453.

Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M. and Forster, R. (2000) 'Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production', *J Exp Med*, 192(11), pp. 1545-52.

Bretscher, P. and Cohn, M. (1970) 'A theory of self-nonself discrimination', *Science*, 169(3950), pp. 1042-9.

Britschgi, M., Steiner, U. C., Schmid, S., Depta, J. P. H., Senti, G., Bircher, A., Burkhart, C., Yawalkar, N. and Pichler, W. J. (2001) 'T-cell involvement in drug-induced acute

generalized exanthematous pustulosis', *The Journal of Clinical Investigation*, 107(11), pp. 1433-1441.

Brockow, K., Przybilla, B., Aberer, W., Bircher, A. J., Brehler, R., Dickel, H., Fuchs, T., Jakob, T., Lange, L., Pfützner, W., Mockenhaupt, M., Ott, H., Pfaar, O., Ring, J., Sachs, B., Sitter, H., Trautmann, A., Treudler, R., Wedi, B., Worm, M., Wurpts, G., Zuberbier, T. and Merk, H. F. (2015) 'Guideline for the diagnosis of drug hypersensitivity reactions: S2K-Guideline of the German Society for Allergology and Clinical Immunology (DGAKI) and the German Dermatological Society (DDG) in collaboration with the Association of German Allergologists (AeDA), the German Society for Pediatric Allergology and Environmental Medicine (GPA), the German Contact Dermatitis Research Group (DKG), the Swiss Society for Allergy and Immunology (SGAI), the Austrian Society for Allergology and Immunology (ÖGAI), the German Academy of Allergology and Environmental Medicine (DAAU), the German Center for Documentation of Severe Skin Reactions and the German Federal Institute for Drugs and Medical Products (BfArM)', *Allergo Journal International*, 24(3), pp. 94-105.

Brody, T. (2018) 'Chapter 7 - Drug–Drug Interactions: Part One (Small Molecule Drugs)', in Brody, T. (ed.) *FDA's Drug Review Process and the Package Label*: Academic Press, pp. 255-335.

Bronson, J., Dhar, M., Ewing, W. and Lonberg, N. (2012) 'Chapter Thirty-One - To Market, To Market—2011', in Desai, M.C. (ed.) *Annual Reports in Medicinal Chemistry*: Academic Press, pp. 499-569.

Brownlie, R. J. and Zamoyska, R. (2013) 'T cell receptor signalling networks: branched, diversified and bounded', *Nat Rev Immunol*, 13(4), pp. 257-69.

Bubp, J., Jen, M. and Matuszewski, K. (2015) 'Caring for glucose-6-phosphate dehydrogenase (G6PD)–deficient patients: Implications for pharmacy', *P and T*, 40(9), pp. 572-574.

Budinger, L. and Hertl, M. (2000) 'Immunologic mechanisms in hypersensitivity reactions to metal ions: an overview', *Allergy*, 55(2), pp. 108-15.

Burkhart, C., Britschgi, M., Strasser, I., Depta, J. P. H., Von Greyerz, S., Barnaba, V. and Pichler, W. J. (2002) 'Non-covalent presentation of sulfamethoxazole to human CD4+ T cells is independent of distinct human leucocyte antigen-bound peptides', *Clinical and Experimental Allergy*, 32(11), pp. 1635-1643.

Burkhart, C., von Greyerz, S., Depta, J. P., Naisbitt, D. J., Britschgi, M., Park, K. B. and Pichler, W. J. (2001) 'Influence of reduced glutathione on the proliferative response of sulfamethoxazole-specific and sulfamethoxazole-metabolite-specific human CD4+ T-cells', *British Journal Of Pharmacology*, 132(3), pp. 623-630.

Burleson, G. R., Burleson, S. C. M. and Burleson, F. G. (2015) 'Chapter 30 - Pulmonary Immunology of Infectious Disease', in Parent, R.A. (ed.) *Comparative Biology of the Normal Lung (Second Edition)*. San Diego: Academic Press, pp. 581-600.

Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V. R., Zhang, H. G., Wang, T., Zheng, J. and Yan, J. (2011) 'Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation', *Immunity*, 35(4), pp. 596-610.

Call, M. E., Pyrdol, J., Wiedmann, M. and Wucherpfennig, K. W. (2002) 'The organizing principle in the formation of the T cell receptor-CD3 complex', *Cell*, 111(7), pp. 967-979.

Callan, H. E., Jenkins, R. E., Maggs, J. L., Lavergne, S. N., Clarke, S. E., Naisbitt, D. J. and Park, B. K. (2009) 'Multiple adduction reactions of nitroso sulfamethoxazole with cysteinyl residues of peptides and proteins: implications for hapten formation', *Chem Res Toxicol*, 22(5), pp. 937-48.

Castrejon, J. L., Berry, N., El-Ghaiesh, S., Gerber, B., Pichler, W. J., Park, B. K. and Naisbitt, D. J. (2010a) 'Stimulation of human T cells with sulfonamides and sulfonamide metabolites', *J Allergy Clin Immunol,* 125(2), pp. 411-418.e4.

Castrejon, J. L., Lavergne, S. N., El-Sheikh, A., Farrell, J., Maggs, J. L., Sabbani, S., O'Neill, P. M., Park, B. K. and Naisbitt, D. J. (2010b) 'Metabolic and chemical origins of cross-reactive immunological reactions to arylamine benzenesulfonamides: T-cell responses to hydroxylamine and nitroso derivatives', *Chemical Research In Toxicology,* 23(1), pp. 184-192.

Cavani, A., Pennino, D. and Eyerich, K. (2012) 'Th17 and Th22 in skin allergy', *Chem Immunol Allergy,* 96, pp. 39-44.

Cawley, J. C. and Hayhoe, F. G. J. (1973) *Ultrastructure of haemic cells : a cytological atlas of normal and leukaemic blood and bone marrow.*

Chalasani, N. P., Hayashi, P. H., Bonkovsky, H. L., Navarro, V. J., Lee, W. M. and Fontana, R. J. (2014) 'ACG Clinical Guideline: the diagnosis and management of idiosyncratic drug-induced liver injury', *Am J Gastroenterol,* 109(7), pp. 950-66; quiz 967.

Chan, N., Li, S. and Perez, E. (2016) 'Chapter 61 - Interactions between Chinese Nutraceuticals and Western Medicines', in Gupta, R.C. (ed.) *Nutraceuticals*. Boston: Academic Press, pp. 875-882.

Chang, J. T., Wherry, E. J. and Goldrath, A. W. (2014) 'Molecular regulation of effector and memory T cell differentiation', *Nat Immunol,* 15(12), pp. 1104-15.

Chaplin, D. D. (2006) '1. Overview of the human immune response', *Journal of Allergy and Clinical Immunology,* 117(2, Supplement 2), pp. S430-S435.

Chaplin, D. D. (2010) 'Overview of the Immune Response', *The Journal of allergy and clinical immunology,* 125(2 Suppl 2), pp. S3-23.

Chen, L. and Flies, D. B. (2013) 'Molecular mechanisms of T cell co-stimulation and co-inhibition', *Nature reviews. Immunology,* 13(4), pp. 227-242.

Chen, P., Lin, J. J., Lu, C. S., Ong, C. T., Hsieh, P. F., Yang, C. C., Tai, C. T., Wu, S. L., Lu, C. H., Hsu, Y. C., Yu, H. Y., Ro, L. S., Lu, C. T., Chu, C. C., Tsai, J. J., Su, Y. H., Lan, S. H., Sung, S. F., Lin, S. Y., Chuang, H. P., Huang, L. C., Chen, Y. J., Tsai, P. J., Liao, H. T., Lin, Y. H., Chen, C. H., Chung, W. H., Hung, S. I., Wu, J. Y., Chang, C. F., Chen, L., Chen, Y. T. and Shen, C. Y. (2011) 'Carbamazepine-induced toxic effects and HLA-B*1502 screening in Taiwan', *N Engl J Med,* 364(12), pp. 1126-33.

Chen, W. T., Wang, C. W., Lu, C. W., Chen, C. B., Lee, H. E., Hung, S. I., Choon, S. E., Yang, C. H., Liu, M. T., Chen, T. J., Fan, W. L., Su, S. C., Lin, Y. Y., Chang, Y. C. and Chung, W. H. (2018) 'The Function of HLA-B*13:01 Involved in the Pathomechanism of Dapsone-Induced Severe Cutaneous Adverse Reactions', *J Invest Dermatol,* 138(7), pp. 1546-1554.

Chessman, D., Kostenko, L., Lethborg, T., Purcell, A. W., Williamson, N. A., Chen, Z., Kjer-Nielsen, L., Mifsud, N. A., Tait, B. D., Holdsworth, R., Almeida, C. A., Nolan, D., Macdonald, W. A., Archbold, J. K., Kellerher, A. D., Marriott, D., Mallal, S., Bharadwaj, M., Rossjohn, J. and McCluskey, J. (2008) 'Human leukocyte antigen class I-restricted activation of CD8+ T cells provides the immunogenetic basis of a systemic drug hypersensitivity', *Immunity,* 28(6), pp. 822-32.

Chien, Y. H., Meyer, C. and Bonneville, M. (2014) 'gammadelta T cells: first line of defense and beyond', *Annu Rev Immunol,* 32, pp. 121-55.

Chipinda, I., Hettick, J. M. and Siegel, P. D. (2011) 'Haptenation: chemical reactivity and protein binding', *Journal of allergy,* 2011, pp. 839682-839682.

Cho, T. and Uetrecht, J. (2017) 'How Reactive Metabolites Induce an Immune Response That Sometimes Leads to an Idiosyncratic Drug Reaction', *Chemical Research in Toxicology,* 30(1), pp. 295-314.

Cho, Y.-T., Yang, C.-W. and Chu, C.-Y. (2017) 'Drug Reaction with Eosinophilia and Systemic Symptoms (DRESS): An Interplay among Drugs, Viruses, and Immune System', *International journal of molecular sciences,* 18(6), pp. 1243.

Choi, Y. S., Kageyama, R., Eto, D., Escobar, T. C., Johnston, R. J., Monticelli, L., Lao, C. and Crotty, S. (2011) 'ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6', *Immunity,* 34(6), pp. 932-946.

Choo, S. Y. (2007) 'The HLA System: Genetics, Immunology, Clinical Testing, and Clinical Implications', *Yonsei Medical Journal,* 48(1), pp. 11-23.

Choudhuri, K., Kearney, A., Bakker, T. R. and van der Merwe, P. A. (2005) 'Immunology: How Do T Cells Recognize Antigen?', *Current Biology,* 15(10), pp. R382-R385.

Choudhuri, K. and van der Merwe, P. A. (2007) 'Molecular mechanisms involved in T cell receptor triggering', *Seminars in Immunology,* 19(4), pp. 255-261.

Chung, W.-H., Hung, S.-I. and Chen, Y.-T. (2007) 'Human leukocyte antigens and drug hypersensitivity', *Current Opinion In Allergy And Clinical Immunology,* 7(4), pp. 317-323.

Chung, W.-H., Hung, S.-I., Hong, H.-S., Hsih, M.-S., Yang, L.-C., Ho, H.-C., Wu, J.-Y. and Chen, Y.-T. (2004a) 'A marker for Stevens–Johnson syndrome', *Nature,* 428, pp. 486.

Chung, W. H., Hung, S. I., Hong, H. S., Hsih, M. S., Yang, L. C., Ho, H. C., Wu, J. Y. and Chen, Y. T. (2004b) 'Medical genetics: a marker for Stevens-Johnson syndrome', *Nature,* 428(6982), pp. 486.

Cialdai, C., Giuliani, S., Valenti, C., Tramontana, M. and Maggi, C. A. (2010) 'Differences between zofenopril and ramipril, two ACE inhibitors, on cough induced by citric acid in guinea pigs: role of bradykinin and PGE2', *Naunyn Schmiedebergs Arch Pharmacol,* 382(5-6), pp. 455-61.

Clark, R. A. F., Ghosh, K. and Tonnesen, M. G. (2007) 'Tissue Engineering for Cutaneous Wounds', *Journal of Investigative Dermatology,* 127(5), pp. 1018-1029.

Coico, R. and Sunshine, G. (2015) *Immunology. [electronic book] : a short course. Online access with subscription: Proquest Ebook Central*: West Sussex, England : Wiley Blackwell, 2015.

Seventh edition.

Cole, J., Aberdein, J., Jubrail, J. and Dockrell, D. H. (2014) 'Chapter Four - The Role of Macrophages in the Innate Immune Response to Streptococcus pneumoniae and Staphylococcus aureus: Mechanisms and Contrasts', in Poole, R.K. (ed.) *Advances in Microbial Physiology*: Academic Press, pp. 125-202.

Cooper, J. A. and Qian, H. (2008) 'A mechanism for SRC kinase-dependent signaling by noncatalytic receptors', *Biochemistry,* 47(21), pp. 5681-5688.

Corthay, A. (2006) 'A Three-cell Model for Activation of Naïve T Helper Cells', *Scandinavian Journal of Immunology,* 64(2), pp. 93-96.

Cote, B., Wechsler, J., Bastuji-Garin, S., Assier, H., Revuz, J. and Roujeau, J. C. (1995) 'Clinicopathologic correlation in erythema multiforme and Stevens-Johnson syndrome', *Arch Dermatol,* 131(11), pp. 1268-72.

Cresswell, P. (2012) 'Intracellular events regulating cross-presentation', *Frontiers in Immunology,* 3(138).

Cribb, A. E., Miller, M., Leeder, J. S., Hill, J. and Spielberg, S. P. (1991) 'Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione. Implications for idiosyncratic toxicity', *Drug Metabolism And Disposition: The Biological Fate Of Chemicals,* 19(5), pp. 900-906.

Cribb, A. E. and Spielberg, S. P. (1992) 'Sulfamethoxazole is metabolized to the hydroxylamine in humans', *Clin Pharmacol Ther,* 51(5), pp. 522-6.

Crotty, S. 2011. Follicular Helper CD4 T cells (T FH). *Annual Review of Immunology*.

Crotty, S. (2014) 'T follicular helper cell differentiation, function, and roles in disease', *Immunity,* 41(4), pp. 529-42.

Curtsinger, J. M., Schmidt, C. S., Mondino, A., Lins, D. C., Kedl, R. M., Jenkins, M. K. and Mescher, M. F. (1999) 'Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells', *J Immunol,* 162(6), pp. 3256-62.

Daly, A. K. and Day, C. P. (2012) 'Genetic association studies in drug-induced liver injury', *Drug Metab Rev,* 44(1), pp. 116-26.

Daly, A. K., Donaldson, P. T., Bhatnagar, P., Shen, Y., Pe'er, I., Floratos, A., Daly, M. J., Goldstein, D. B., John, S., Nelson, M. R., Graham, J., Park, B. K., Dillon, J. F., Bernal, W., Cordell, H. J., Pirmohamed, M., Aithal, G. P. and Day, C. P. (2009) 'HLA-B*5701 genotype is a major determinant of drug-induced liver injury due to flucloxacillin', *Nat Genet,* 41(7), pp. 816-9.

Danilova, N. (2006) 'The evolution of immune mechanisms', *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution,* 306(6), pp. 496-520.

Das, A., Sinha, M., Datta, S., Abas, M., Chaffee, S., Sen, C. K. and Roy, S. (2015) 'Monocyte and Macrophage Plasticity in Tissue Repair and Regeneration', *The American Journal of Pathology,* 185(10), pp. 2596-2606.

Dasgupta, A. and Wahed, A. (2014) 'Chapter 24 - Autoimmunity, Complement, and Immunodeficiency', in Dasgupta, A. & Wahed, A. (eds.) *Clinical Chemistry, Immunology and Laboratory Quality Control*. San Diego: Elsevier, pp. 427-447.

Davey, M. S., Willcox, C. R., Joyce, S. P., Ladell, K., Kasatskaya, S. A., McLaren, J. E., Hunter, S., Salim, M., Mohammed, F., Price, D. A., Chudakov, D. M. and Willcox, B. E. (2017) 'Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance', *Nature Communications,* 8, pp. 14760.

David, M., Jonathan, B., David, B. R. and Ivan, M. R. (2013) *IMMUNOLOGY*. Elsevier Ltd. Reprint, Eighth edition.

David, M., Jonathan, B., David, R. and Ivan, R. (2012) 'Immunology'. 8th edition ed.

Davies, H. (1997) *introductory immunobiology.* 1st edition edn.: CHAPMAN&HALL.

De Rosa, S. C., Roederer, M., Herzenberg, L. A. and Herzenberg, L. A. (2001) '11-color, 13-parameter flow cytometry: Identification of human naive T cells by phenotype, function, and T-cell receptor diversity', *Nature Medicine,* 7(2), pp. 245-248.

Degoot, A. M., Chirove, F. and Ndifon, W. (2018) 'Trans-Allelic Model for Prediction of Peptide:MHC-II Interactions', *Frontiers in Immunology,* 9(1410).

Denzin Lisa, K., Fallas Jennifer, L., Prendes, M. and Yi, W. (2005) 'Right place, right time, right peptide: DO keeps DM focused', *Immunological Reviews,* 207(1), pp. 279-292.

Depta, J. P., Altznauer, F., Gamerdinger, K., Burkhart, C., Weltzien, H. U. and Pichler, W. J. (2004) 'Drug interaction with T-cell receptors: T-cell receptor density determines degree of cross-reactivity', *J Allergy Clin Immunol,* 113(3), pp. 519-27.

Dixon, M. B. and Lien, Y. H. (2008) 'Tolvaptan and its potential in the treatment of hyponatremia', *Therapeutics and Clinical Risk Management,* 4(6), pp. 1149-1155.

Drago, F., Cogorno, L., Broccolo, F., Ciccarese, G. and Parodi, A. (2016) 'A fatal case of DRESS induced by strontium ranelate associated with HHV-7 reactivation', *Osteoporos Int,* 27(3), pp. 1261-1264.

Du, W., Tutag Lehr, V., Caverly, M., Kelm, L., Reeves, J. and Lieh-Lai, M. (2013) 'Incidence and Costs of Adverse Drug Reactions in a Tertiary Care Pediatric Intensive Care Unit', *The Journal of Clinical Pharmacology,* 53(5), pp. 567-573.

Duhen, T., Geiger, R., Jarrossay, D., Lanzavecchia, A. and Sallusto, F. (2009) 'Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells', *Nat Immunol,* 10(8), pp. 857-63.

Eagar, T. N. and Miller, S. D. (2008) '17 - Helper T-cell subsets and control of the inflammatory response A2 - Rich, Robert R', in Fleisher, T.A., Shearer, W.T., Schroeder, H.W., Frew, A.J. & Weyand, C.M. (eds.) *Clinical Immunology (Third Edition)*. Edinburgh: Mosby, pp. 259-270.

Ebert, L. M., Schaerli, P. and Moser, B. (2005) 'Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues', *Mol Immunol,* 42(7), pp. 799-809.

Edwards, I. R. and Aronson, J. K. (2000) 'Adverse drug reactions: definitions, diagnosis, and management', *The Lancet,* 356(9237), pp. 1255-1259.

El-Ghaiesh, S., Sanderson, J. P., Farrell, J., Lavergne, S. N., Syn, W. K., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2011) 'Trimethoprim stimulates T-cells through metabolism-dependent and -independent pathways', *Chem Res Toxicol,* 24(6), pp. 791-3.

Ellis, M. K., Hill, S. and Foster, P. M. (1992) 'Reactions of nitrosonitrobenzenes with biological thiols: identification and reactivity of glutathion-S-yl conjugates', *Chemico-Biological Interactions,* 82(2), pp. 151-163.

Elsheikh, A., Castrejon, L., Lavergne, S. N., Whitaker, P., Monshi, M., Callan, H., El-Ghaiesh, S., Farrell, J., Pichler, W. J., Peckham, D., Park, B. K. and Naisbitt, D. J. (2011) 'Enhanced antigenicity leads to altered immunogenicity in sulfamethoxazole-hypersensitive patients with cystic fibrosis', *Journal of Allergy and Clinical Immunology,* 127(6), pp. 1543-1551.e3.

Elsheikh, A., N Lavergne, S., Luis Castrejon, J., Farrell, J., Wang, H., Sathish, J., J Pichler, W., Park, B. and Naisbitt, D. (2010) *Drug Antigenicity, Immunogenicity, and Costimulatory Signaling: Evidence for Formation of a Functional Antigen through Immune Cell Metabolism.*

Eric, E. S. (2018) 'Hyponatremia', *Medscape*.

Erkes, D. A. and Selvan, S. R. (2014) 'Hapten-induced contact hypersensitivity, autoimmune reactions, and tumor regression: plausibility of mediating antitumor immunity', *Journal of immunology research,* 2014, pp. 175265-175265.

Eyerich, S., Eyerich, K., Cavani, A. and Schmidt-Weber, C. (2010) 'IL-17 and IL-22: siblings, not twins', *Trends In Immunology,* 31(9), pp. 354-361.

Eyerich, S., Eyerich, K., Pennino, D., Carbone, T., Nasorri, F., Pallotta, S., Cianfarani, F., Odorisio, T., Traidl-Hoffmann, C., Behrendt, H., Durham, S. R., Schmidt-Weber, C. B. and Cavani, A. (2009) 'Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling', *J Clin Invest,* 119(12), pp. 3573-85.

Falcone, F. H., Haas, H. and Gibbs, B. F. (2000) 'The human basophil: a new appreciation of its role in immune responses', *Blood,* 96(13), pp. 4028-38.

Fan, W.-L., Shiao, M.-S., Hui, R. C.-Y., Su, S.-C., Wang, C.-W., Chang, Y.-C. and Chung, W.-H. (2017) 'HLA Association with Drug-Induced Adverse Reactions', *Journal of immunology research,* 2017, pp. 3186328-3186328.

Faulkner, L., Martinsson, K., Santoyo-Castelazo, A., Cederbrant, K., Schuppe-Koistinen, I., Powell, H., Tugwood, J., Naisbitt, D. J. and Park, B. K. (2012a) 'The Development of In Vitro Culture Methods to Characterize Primary T-Cell Responses to Drugs', *Toxicological Sciences,* 127(1), pp. 150-158.

Faulkner, L., Martinsson, K., Santoyo-Castelazo, A., Cederbrant, K., Schuppe-Koistinen, I., Powell, H., Tugwood, J., Naisbitt, D. J. and Park, B. K. (2012b) 'The development of in vitro culture methods to characterize primary T-cell responses to drugs', *Toxicol Sci,* 127(1), pp. 150-8.

Faulkner, L., Meng, X., Park, B. K. and Naisbitt, D. J. (2014) 'The importance of hapten-protein complex formation in the development of drug allergy', *Curr Opin Allergy Clin Immunol,* 14(4), pp. 293-300.

Fazilleau, N., Mark, L., McHeyzer-Williams, L. J. and McHeyzer-Williams, M. G. (2009) 'Follicular helper T cells: lineage and location', *Immunity,* 30(3), pp. 324-35.

Fazilleau, N., McHeyzer-Williams, L. J. and McHeyzer-Williams, M. G. (2007) 'Local development of effector and memory T helper cells', *Curr Opin Immunol,* 19(3), pp. 259-67.

Ferner, R. E. and Butt, T. F. (2008) 'Adverse drug reactions', *Medicine,* 36(7), pp. 364-368.

Finney, D. J. (2006) *Writings on Pharmacovigilance.* Selected Articles. Uppsala: The Uppsala Monitoring Centre.

.

Flaherty, D. K. (2012a) 'Chapter 20 - Cytotoxic T Cells', in Flaherty, D.K. (ed.) *Immunology for Pharmacy*. Saint Louis: Mosby, pp. 162-168.

Flaherty, D. K. (2012b) *Immunology for pharmacy. [electronic book]. Online access with subscription: Elsevier (Sciencedirect Freedom Collection)*: St. Louis, Mo. : Elsevier/Mosby, 2012.

Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y. (2003) 'Foxp3 programs the development and function of CD4+CD25+ regulatory T cells', *Nat Immunol,* 4(4), pp. 330-6.

Formica, D., Sultana, J., Cutroneo, P. M., Lucchesi, S., Angelica, R., Crisafulli, S., Ingrasciotta, Y., Salvo, F., Spina, E. and Trifiro, G. (2018) 'The economic burden of preventable adverse drug reactions: a systematic review of observational studies', *Expert Opin Drug Saf,* 17(7), pp. 681-695.

Fowell, A. J. and Nash, K. L. (2010a) 'Telaprevir: a new hope in the treatment of chronic hepatitis C?', (no. 8), pp. 512.

Fowell, A. J. and Nash, K. L. (2010b) 'Telaprevir: a new hope in the treatment of chronic hepatitis C?', *Advances in Therapy,* 27(8), pp. 512-522.

Francisco, L. M., Salinas, V. H., Brown, K. E., Vanguri, V. K., Freeman, G. J., Kuchroo, V. K. and Sharpe, A. H. (2009) 'PD-L1 regulates the development, maintenance, and function of induced regulatory T cells', *J Exp Med,* 206(13), pp. 3015-29.

Frederick, M. 2000. Immunobiology: The Immune System in Health and Disease Charles A. Janeway, Jr. Paul Travers Mark Walport J. Donald Capra. University of Chicago Press.

Freeman, J. D., Warren, R. L., Webb, J. R., Nelson, B. H. and Holt, R. A. (2009) 'Profiling the T-cell receptor beta-chain repertoire by massively parallel sequencing', *Genome Research,* 19(10), pp. 1817-1824.

Galli, S. L., CS. (1999) *Allergy. In Fundamental Immunology,*. 4th edition edn.

Gallucci, S. and Matzinger, P. (2001) 'Danger signals: SOS to the immune system', *Current Opinion in Immunology,* 13(1), pp. 114-119.

Garg, V., Kauffman, R. S., Beaumont, M. and van Heeswijk, R. P. (2012) 'Telaprevir: pharmacokinetics and drug interactions', *Antivir Ther,* 17(7), pp. 1211-21.

Gargani, Y. and Kitchen, G. (2012) *Crash Course Haematology and Immunology E-Book.* Mosby Ltd.

Ghosh, K., Banerjee, G., Ghosal, A. K. and Nandi, J. (2011) 'CUTANEOUS DRUG HYPERSENSITIVITY: IMMUNOLOGICAL AND GENETIC PERSPECTIVE', *Indian Journal of Dermatology,* 56(2), pp. 137-144.

Gibson, A., Faulkner, L., Wood, S., Park, B. K. and Naisbitt, D. J. (2017) 'Identification of drug- and drug-metabolite immune responses originating from both naive and memory T cells', *J Allergy Clin Immunol,* 140(2), pp. 578-581.e5.

Gibson, A., Ogese, M., Sullivan, A., Wang, E., Saide, K., Whitaker, P., Peckham, D., Faulkner, L., Park, B. K. and Naisbitt, D. J. (2014) 'Negative regulation by PD-L1 during drug-specific priming of IL-22-secreting T cells and the influence of PD-1 on effector T cell function', *Journal Of Immunology (Baltimore, Md.: 1950),* 192(6), pp. 2611-2621.

Gleich, G. J. and Loegering, D. A. (1984) 'Immunobiology of eosinophils', *Annu Rev Immunol,* 2, pp. 429-59.

Godfrey, D. I., McCluskey, J. and Rossjohn, J. (2005) 'CD1d antigen presentation: treats for NKT cells', *Nature Immunology,* 6, pp. 754.

Goldsby and Richard, A. (2003) *Immunology.* New York : W.H. Freeman, 2003.

5th ed.

Gomes, E. R. and Demoly, P. (2005) 'Epidemiology of hypersensitivity drug reactions', *Curr Opin Allergy Clin Immunol,* 5(4), pp. 309-16.

Gómez, E., Torres, M. J., Mayorga, C. and Blanca, M. (2012) 'Immunologic Evaluation of Drug Allergy', *Allergy, Asthma & Immunology Research,* 4(5), pp. 251-263.

Good, M., Kolls, J. K. and Empey, K. M. (2017) '130 - Neonatal Pulmonary Host Defense', in Polin, R.A., Abman, S.H., Rowitch, D.H., Benitz, W.E. & Fox, W.W. (eds.) *Fetal and Neonatal Physiology (Fifth Edition)*: Elsevier, pp. 1262-1293.e12.

Goronzy, J. J. and Weyand, C. M. (2008) 'T-cell co-stimulatory pathways in autoimmunity', *Arthritis Research & Therapy,* 10(Suppl 1), pp. S3-S3.

Grant, D. M., Kalant, H. and Mitchell, J. (2007) *Principles of medical pharmacology.* Toronto : Saunders Elsevier, 2007.

7th ed.

Graziano, F. M., Cook, E. B. and Stahl, J. L. (1999) 'Cytokines, chemokines, RANTES, and eotaxin', *Allergy Asthma Proc,* 20(3), pp. 141-6.

Gruchalla, R. S. (2001) 'Drug metabolism, danger signals, and drug-induced hypersensitivity', *Journal of Allergy and Clinical Immunology,* 108(4), pp. 475-488.

Guermonprez, P., Valladeau, J., Zitvogel, L., Thery, C. and Amigorena, S. (2002) 'Antigen presentation and T cell stimulation by dendritic cells', *Annu Rev Immunol,* 20, pp. 621-67.

Hamann, D., Baars, P. A., Rep, M. H., Hooibrink, B., Kerkhof-Garde, S. R., Klein, M. R. and van Lier, R. A. (1997) 'Phenotypic and functional separation of memory and effector human CD8+ T cells', *J Exp Med,* 186(9), pp. 1407-18.

Harrington, L. E. (2019) '8 - T-Cell Development', in Rich, R.R., Fleisher, T.A., Shearer, W.T., Schroeder, H.W., Frew, A.J. & Weyand, C.M. (eds.) *Clinical Immunology (Fifth Edition)*. London: Content Repository Only!, pp. 119-125.e1.

Hay, M., Thomas, D. W., Craighead, J. L., Economides, C. and Rosenthal, J. (2014) 'Clinical development success rates for investigational drugs', *Nat Biotechnol,* 32(1), pp. 40-51.

Hayday, A. and Pao, W. (1998) 'T Cell Receptor, γδ', in Delves, P.J. (ed.) *Encyclopedia of Immunology (Second Edition)*. Oxford: Elsevier, pp. 2268-2278.

Herbelin, A., Gombert, J. M., Lepault, F., Bach, J. F. and Chatenoud, L. (1998) 'Mature mainstream TCR alpha beta+CD4+ thymocytes expressing L-selectin mediate "active tolerance" in the nonobese diabetic mouse', *J Immunol,* 161(5), pp. 2620-8.

Hewitt, E. W. (2003) 'The MHC class I antigen presentation pathway: strategies for viral immune evasion', *Immunology,* 110(2), pp. 163-169.

Hoffman, W., Lakkis, F. G. and Chalasani, G. (2016) 'B Cells, Antibodies, and More', *Clinical Journal of the American Society of Nephrology : CJASN,* 11(1), pp. 137-154.

Homey, B., Alenius, H., Muller, A., Soto, H., Bowman, E. P., Yuan, W., McEvoy, L., Lauerma, A. I., Assmann, T., Bunemann, E., Lehto, M., Wolff, H., Yen, D., Marxhausen, H., To, W., Sedgwick, J., Ruzicka, T., Lehmann, P. and Zlotnik, A. (2002) 'CCL27-CCR10 interactions regulate T cell-mediated skin inflammation', *Nat Med*, 8(2), pp. 157-65.

Honda, T., Egawa, G., Grabbe, S. and Kabashima, K. (2013) 'Update of immune events in the murine contact hypersensitivity model: toward the understanding of allergic contact dermatitis', *J Invest Dermatol*, 133(2), pp. 303-15.

Hoofnagle, J. H. (2002) 'Course and outcome of hepatitis C', *Hepatology*, 36(5 Suppl 1), pp. S21-9.

Hori, S., Nomura, T. and Sakaguchi, S. (2003) 'Control of regulatory T cell development by the transcription factor Foxp3', *Science*, 299(5609), pp. 1057-61.

Huang, Y. S., Chern, H. D., Su, W. J., Wu, J. C., Lai, S. L., Yang, S. Y., Chang, F. Y. and Lee, S. D. (2002) 'Polymorphism of the N-acetyltransferase 2 gene as a susceptibility risk factor for antituberculosis drug-induced hepatitis', *Hepatology*, 35(4), pp. 883-9.

Hung, S. I., Chung, W. H., Liou, L. B., Chu, C. C., Lin, M., Huang, H. P., Lin, Y. L., Lan, J. L., Yang, L. C., Hong, H. S., Chen, M. J., Lai, P. C., Wu, M. S., Chu, C. Y., Wang, K. H., Chen, C. H., Fann, C. S., Wu, J. Y. and Chen, Y. T. (2005) 'HLA-B*5801 allele as a genetic marker for severe cutaneous adverse reactions caused by allopurinol', *Proc Natl Acad Sci U S A*, 102(11), pp. 4134-9.

Iezzi, G., Boni, A., Degl'Innocenti, E., Grioni, M., Bertilaccio, M. T. and Bellone, M. (2006) 'Type 2 cytotoxic T lymphocytes modulate the activity of dendritic cells toward type 2 immune responses', *J Immunol*, 177(4), pp. 2131-7.

Illing, P. T., Vivian, J. P., Dudek, N. L., Kostenko, L., Chen, Z., Bharadwaj, M., Miles, J. J., Kjer-Nielsen, L., Gras, S., Williamson, N. A., Burrows, S. R., Purcell, A. W., Rossjohn, J. and McCluskey, J. (2012) 'Immune self-reactivity triggered by drug-modified HLA-peptide repertoire', *Nature*, 486(7404), pp. 554-8.

Illing, P. T., Vivian, J. P., Purcell, A. W., Rossjohn, J. and McCluskey, J. (2013) 'Human leukocyte antigen-associated drug hypersensitivity', *Curr Opin Immunol*, 25(1), pp. 81-9.

Jain, S. (2014) 'Pathogenesis of Chronic Urticaria: An Overview', *Dermatology Research and Practice*, 2014, pp. 674709.

Jandl, C., Loetsch, C. and King, C. (2017) 'Cytokine Expression by T Follicular Helper Cells', in Calado, D.P. (ed.) *Germinal Centers: Methods and Protocols*. New York, NY: Springer New York, pp. 95-103.

Janeway, C. (2001) *Immunobiology 5. [electronic book] : the immune system in health and disease. Free online access: Pubmed*: New York : Garland Pub., c2001.

5th ed.

Janeway, C. J., Travers, P., Walport, M. and al., e. (2001) *Immunobiology: The Immune System in Health and Disease.* 5th edition edn. New York: Garland Science.

Jesudian, A. B., Gambarin-Gelwan, M. and Jacobson, I. M. (2012) 'Advances in the treatment of hepatitis C virus infection', *Gastroenterol Hepatol (N Y)*, 8(2), pp. 91-101.

Jia, L. and Wu, C. (2014) 'The biology and functions of Th22 cells', *Adv Exp Med Biol*, 841, pp. 209-30.

Johansson, S. G., Bieber, T., Dahl, R., Friedmann, P. S., Lanier, B. Q., Lockey, R. F., Motala, C., Ortega Martell, J. A., Platts-Mills, T. A., Ring, J., Thien, F., Van Cauwenberge, P. and Williams, H. C. (2004) 'Revised nomenclature for allergy for global use:

Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003', *J Allergy Clin Immunol,* 113(5), pp. 832-6.

Johnston, R. J., Poholek, A. C., DiToro, D., Yusuf, I., Eto, D., Barnett, B., Dent, A. L., Craft, J. and Crotty, S. (2009) 'Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation', *Science,* 325(5943), pp. 1006-10.

Kaech, S. M. and Cui, W. (2012) 'Transcriptional control of effector and memory CD8+ T cell differentiation', *Nat Rev Immunol,* 12(11), pp. 749-61.

Kagami, S., Rizzo, H. L., Lee, J. J., Koguchi, Y. and Blauvelt, A. (2010) 'Circulating Th17, Th22, and Th1 cells are increased in psoriasis', *J Invest Dermatol,* 130(5), pp. 1373-83.

Kaplan, M. H., Hufford, M. M. and Olson, M. R. (2015) 'The development and in vivo function of T helper 9 cells', *Nat Rev Immunol,* 15(5), pp. 295-307.

Kaplowitz, N. (2002) 'Biochemical and cellular mechanisms of toxic liver injury', *Semin Liver Dis,* 22(2), pp. 137-44.

Kaplowitz, N. (2004) 'Drug-Induced Liver Injury', *Clinical Infectious Diseases,* 38(Supplement_2), pp. S44-S48.

Karre, K., Ljunggren, H. G., Piontek, G. and Kiessling, R. (1986) 'Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy', *Nature,* 319(6055), pp. 675-8.

Kenna, J. G., Knight, T. L. and van Pelt, F. N. (1993) 'Immunity to halothane metabolite-modified proteins in halothane hepatitis', *Ann N Y Acad Sci,* 685, pp. 646-61.

Kenneth Murphy, C. W. (2017) *Janeway's Immunobiology.*

Khan, M. M. (2008) *Immunopharmacology. [electronic book]. Online access with purchase: Springer*: New York : Springer, c2008.

Khan, M. M. (2016) *Immunopharmacology. [electronic book]. Online access with purchase: Springer*: Switzerland : Springer, [2016]

Second edition.

Kim, C. H., Rott, L., Kunkel, E. J., Genovese, M. C., Andrew, D. P., Wu, L. and Butcher, E. C. (2001a) 'Rules of chemokine receptor association with T cell polarization in vivo', *J Clin Invest,* 108(9), pp. 1331-9.

Kim, C. H., Rott, L. S., Clark-Lewis, I., Campbell, D. J., Wu, L. and Butcher, E. C. (2001b) 'Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells', *J Exp Med,* 193(12), pp. 1373-81.

Kim, S. H., Saide, K., Farrell, J., Faulkner, L., Tailor, A., Ogese, M., Daly, A. K., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2015) 'Characterization of amoxicillin- and clavulanic acid-specific T cells in patients with amoxicillin-clavulanate-induced liver injury', *Hepatology,* 62(3), pp. 887-99.

Kindmark, A., Jawaid, A., Harbron, C. G., Barratt, B. J., Bengtsson, O. F., Andersson, T. B., Carlsson, S., Cederbrant, K. E., Gibson, N. J., Armstrong, M., Lagerstrom-Fermer, M. E., Dellsen, A., Brown, E. M., Thornton, M., Dukes, C., Jenkins, S. C., Firth, M. A., Harrod, G. O., Pinel, T. H., Billing-Clason, S. M., Cardon, L. R. and March, R. E. (2008) 'Genome-wide pharmacogenetic investigation of a hepatic adverse event without clinical signs of immunopathology suggests an underlying immune pathogenesis', *Pharmacogenomics J,* 8(3), pp. 186-95.

King, C. (2009) 'New insights into the differentiation and function of T follicular helper cells', *Nature Reviews Immunology,* 9(11), pp. 757-766.

Kinne, R. W., Stuhlmüller, B. and Burmester, G. R. (2009) 'CHAPTER 8C - Macrophages', in Hochberg, M.C., Silman, A.J., Smolen, J.S., Weinblatt, M.E. & Weisman, M.H. (eds.) *Rheumatoid Arthritis*. Philadelphia: Mosby, pp. 107-115.

Kita, H. (1996) 'The eosinophil: a cytokine-producing cell?', *J Allergy Clin Immunol,* 97(4), pp. 889-92.

Ko, T. M., Chung, W. H., Wei, C. Y., Shih, H. Y., Chen, J. K., Lin, C. H., Chen, Y. T. and Hung, S. I. (2011) 'Shared and restricted T-cell receptor use is crucial for carbamazepine-induced Stevens-Johnson syndrome', *J Allergy Clin Immunol,* 128(6), pp. 1266-1276.e11.

Koenderman, L., Buurman, W. and Daha, M. R. (2014) 'The innate immune response', *Immunology Letters,* 162(2, Part B), pp. 95-102.

Koshy, P. J., Henderson, N., Logan, C., Life, P. F., Cawston, T. E. and Rowan, A. D. (2002) 'Interleukin 17 induces cartilage collagen breakdown: Novel synergistic effects in combination with proinflammatory cytokines', *Annals of the Rheumatic Diseases,* 61(8), pp. 704-713.

Kosseifi, S. G., Guha, B., Nassour, D. N., Chi, D. S. and Krishnaswamy, G. (2006) 'The Dapsone hypersensitivity syndrome revisited: a potentially fatal multisystem disorder with prominent hepatopulmonary manifestations', *J Occup Med Toxicol,* 1, pp. 9.

Kuhns, M. S., Davis, M. M. and Garcia, K. C. (2006) 'Deconstructing the form and function of the TCR/CD3 complex', *Immunity,* 24(2), pp. 133-9.

Kurts, C. (2008) 'Th17 cells: a third subset of CD4 + T effector cells involved in organ-specific autoimmunity', *Nephrology Dialysis Transplantation,* 23(3), pp. 816-819.

Kutteh, W. H., Stovall, D. W. and Schust, D. J. (2014) 'Chapter 14 - Immunology and Reproduction A2 - Strauss, Jerome F', in Barbieri, R.L. (ed.) *Yen & Jaffe's Reproductive Endocrinology (Seventh Edition)*. Philadelphia: W.B. Saunders, pp. 287-307.e3.

Lafferty, K. J. and Cunningham, A. J. (1975) 'A new analysis of allogeneic interactions', *Aust J Exp Biol Med Sci,* 53(1), pp. 27-42.

Lahita, R. G. (2007) 'Chapter 44 - Nonsteroid Treatment of Systemic Lupus Erythematosus A2 - Tsokos, George C', in Gordon, C. & Smolen, J.S. (eds.) *Systemic Lupus Erythematosus*. Philadelphia: Mosby, pp. 483-486.

Lai, A. Y. and Kondo, M. (2008) 'T and B lymphocyte differentiation from hematopoietic stem cell', *Seminars in immunology,* 20(4), pp. 207-212.

Landsteiner, K. and Jacobs, J. (1935) 'STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS', *The Journal of Experimental Medicine,* 61(5), pp. 643-656.

Lang, L. (2007) 'Interim results presented at EASL from PROVE 1 clinical trial of investigational drug telaprevir in patients with genotype 1 hepatitis C', *Gastroenterology,* 132(7), pp. 2283-4.

Larosa, D. F. and Orange, J. S. (2008) '1. Lymphocytes', *J Allergy Clin Immunol,* 121(2 Suppl), pp. S364-9; quiz S412.

Larrey, D. (2002) 'Epidemiology and individual susceptibility to adverse drug reactions affecting the liver', *Semin Liver Dis,* 22(2), pp. 145-55.

Lazarou, J., Pomeranz, B. H. and Corey, P. N. (1998) 'Incidence of adverse drug reactions in hospitalized patients: a meta-analysis of prospective studies', *Jama,* 279(15), pp. 1200-5.

Leckband, S. G., Kelsoe, J. R., Dunnenberger, H. M., George, A. L., Jr., Tran, E., Berger, R., Muller, D. J., Whirl-Carrillo, M., Caudle, K. E. and Pirmohamed, M. (2013) 'Clinical Pharmacogenetics Implementation Consortium guidelines for HLA-B genotype and carbamazepine dosing', *Clin Pharmacol Ther,* 94(3), pp. 324-8.

Leder, P. (1982) 'The genetics of antibody diversity', *Sci Am,* 246(5), pp. 102-15.

Lee, J. J. and Rosenberg, H. F. (2013) 'Chapter 15 - Antieosinophil Therapeutics A2 - Lee, James J', in Rosenberg, H.F. (ed.) *Eosinophils in Health and Disease*. Boston: Academic Press, pp. 577-605.

Lee, W. M. (2017) 'Acetaminophen (APAP) hepatotoxicity—Isn't it time for APAP to go away?', *Journal of Hepatology,* 67(6), pp. 1324-1331.

Lee, Y., Awasthi, A., Yosef, N., Quintana, F. J., Xiao, S., Peters, A., Wu, C., Kleinewietfeld, M., Kunder, S., Hafler, D. A., Sobel, R. A., Regev, A. and Kuchroo, V. K. (2012) 'Induction and molecular signature of pathogenic TH17 cells', *Nat Immunol,* 13(10), pp. 991-9.

Lee, Y. N. (2016) 'Chapter 4 - TCR Repertoire Analysis A2 - Tan, Seng-Lai', *Translational Immunology*. Boston: Academic Press, pp. 115-129.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J. M. and Hoffmann, J. A. (1996) 'The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults', *Cell,* 86(6), pp. 973-83.

Levine, B. B. (1960) 'Studies on the mechanism of the formation of the penicillin antigen. I. Delayed allergic cross-reactions among penicillin G and its degradation products', *J Exp Med,* 112, pp. 1131-56.

Levine, B. B. and Price, V. H. (1964) 'Studies on the immunological mechanisms of penicillin allergy: II. Antigenic specificities of allergic wheal-and-flare skin responses in patients with histories of penicillin allergy', *Immunology,* 7(5), pp. 542-556.

Ley, S. C., Tan, K.-N., Kubo, R., Sy, M.-S. and Terhorst, C. (1989) 'Surface expression of CD3 in the absence of T cell receptor (TcR): evidence for sorting of partial TcR/CD3 complexes in a post-endoplasmic reticulum compartment', *European Journal of Immunology,* 19(12), pp. 2309-2317.

Li, J. and Uetrecht, J. P. (2010) 'The Danger Hypothesis Applied to Idiosyncratic Drug Reactions', in Uetrecht, J. (ed.) *Adverse Drug Reactions*. Berlin, Heidelberg: Springer Berlin Heidelberg, pp. 493-509.

Li, M. O. and Rudensky, A. Y. (2016) 'T cell receptor signalling in the control of regulatory T cell differentiation and function', *Nat Rev Immunol,* 16(4), pp. 220-33.

Liang, J., Qu, H., Wang, X., Wang, A., Liu, L., Tu, P., Li, R. and Wang, M. (2018) 'Drug Reaction with Eosinophilia and Systemic Symptoms Associated with Reactivation of Epstein-Barr Virus and/or Cytomegalovirus Leading to Hemophagocytic Syndrome in One of Two Patients', *Annals of dermatology,* 30(1), pp. 71-74.

Lichtenfels, M., Farrell, J., Ogese, M. O., Bell, C. C., Eckle, S., McCluskey, J., Park, B. K., Alfirevic, A., Naisbitt, D. J. and Pirmohamed, M. (2014) 'HLA restriction of carbamazepine-specific T-Cell clones from an HLA-A*31:01-positive hypersensitive patient', *Chem Res Toxicol,* 27(2), pp. 175-7.

Lim, K. G., Wan, H. C., Resnick, M., Wong, D. T., Cruikshank, W. W., Kornfeld, H., Center, D. M. and Weller, P. F. (1995) 'Human eosinophils release the lymphocyte and eosinophil active cytokines, RANTES and lymphocyte chemoattractant factor', *Int Arch Allergy Immunol,* 107(1-3), pp. 342.

Locksley, R. M., Reiner, S. L., Hatam, F., Littman, D. R. and Killeen, N. (1993) 'Helper T cells without CD4: control of leishmaniasis in CD4-deficient mice', *Science,* 261(5127), pp. 1448-51.

Logan, M. R., Odemuyiwa, S. O. and Moqbel, R. (2003) 'Understanding exocytosis in immune and inflammatory cells: the molecular basis of mediator secretion', *J Allergy Clin Immunol,* 111(5), pp. 923-32; quiz 933.

Lu, Y., Slizgi, J. R., Brouwer, K. R., Claire, R. L. S., Freeman, K. M., Pan, M., Brock, W. J. and Brouwer, K. L. (2016) 'Hepatocellular Disposition and Transporter Interactions

with Tolvaptan and Metabolites in Sandwich-Cultured Human Hepatocytes', *Drug Metabolism and Disposition*.

Lucas, A., Lucas, M., Strhyn, A., Keane, N. M., McKinnon, E., Pavlos, R., Moran, E. M., Meyer-Pannwitt, V., Gaudieri, S., D'Orsogna, L., Kalams, S., Ostrov, D. A., Buus, S., Peters, B., Mallal, S. and Phillips, E. (2015) 'Abacavir-reactive memory T cells are present in drug naïve individuals', *Plos One,* 10(2), pp. e0117160-e0117160.

Lyons, M. J., Yoshimura, T. and McMurray, D. N. (2004) 'Interleukin (IL)-8 (CXCL8) induces cytokine expression and superoxide formation by guinea pig neutrophils infected with Mycobacterium tuberculosis', *Tuberculosis (Edinb),* 84(5), pp. 283-92.

Mabtech (2018) 'ELISpot Assay Principle [Online]. '.

Mak, T. W. and Saunders, M. E. (2006) '15 - T Cell Differentiation and Effector Function', in Mak, T.W. & Saunders, M.E. (eds.) *The Immune Response*. Burlington: Academic Press, pp. 403-432.

Mak, T. W., Saunders, M. E. and Jett, B. D. (2014) 'Chapter 3 - Innate Immunity', in Mak, T.W., Saunders, M.E. & Jett, B.D. (eds.) *Primer to the Immune Response (Second Edition)*. Boston: Academic Cell, pp. 55-83.

Male, D., Brostoff, J., Roth, D. and Roitt, I. (2012) *Immunology : With STUDENT CONSULT Online Access*. Edinburgh, UNITED KINGDOM: Elsevier.

Mallal, S., Nolan, D., Witt, C., Masel, G., Martin, A. M., Moore, C., Sayer, D., Castley, A., Mamotte, C., Maxwell, D., James, I. and Christiansen, F. T. (2002) 'Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir', *The Lancet,* 359(9308), pp. 727-732.

Mallal, S., Phillips, E., Carosi, G., Molina, J. M., Workman, C., Tomazic, J., Jagel-Guedes, E., Rugina, S., Kozyrev, O., Cid, J. F., Hay, P., Nolan, D., Hughes, S., Hughes, A., Ryan, S., Fitch, N., Thorborn, D. and Benbow, A. (2008) 'HLA-B*5701 screening for hypersensitivity to abacavir', *N Engl J Med,* 358(6), pp. 568-79.

Mandal, A. and Viswanathan, C. (2015) 'Natural killer cells: In health and disease', *Hematology/Oncology and Stem Cell Therapy,* 8(2), pp. 47-55.

Maria, V. A. and Victorino, R. M. (1997) 'Diagnostic value of specific T cell reactivity to drugs in 95 cases of drug induced liver injury', *Gut,* 41(4), pp. 534-40.

Mathelier-Fusade, P. (2006) 'Drug-induced urticarias', *Clinical Reviews in Allergy & Immunology,* 30(1), pp. 19-23.

Matusiewicz, K., Iwanczak, B. and Matusiewicz, M. (2018) 'Th9 lymphocytes and functions of interleukin 9 with the focus on IBD pathology', *Adv Med Sci,* 63(2), pp. 278-284.

Matzinger, P. (1994) 'Tolerance, danger, and the extended family', *Annu Rev Immunol,* 12, pp. 991-1045.

Matzinger, P. (2002) 'The danger model: a renewed sense of self', *Science,* 296(5566), pp. 301-5.

Maverakis, E., Kim, K., Shimoda, M., Gershwin, M. E., Patel, F., Wilken, R., Raychaudhuri, S., Ruhaak, L. R. and Lebrilla, C. B. (2015) 'Glycans in the immune system and The Altered Glycan Theory of Autoimmunity: a critical review', *Journal of autoimmunity,* 57, pp. 1-13.

Mazzucchelli, R. and Durum, S. K. (2007) 'Interleukin-7 receptor expression: Intelligent design', *Nature Reviews Immunology,* 7(2), pp. 144-154.

McDowell, J. M., Johnson, G. M. and Bradnam, L. V. (2011) 'Towards a neurophysiological mechanisms-based classification of adverse reactions to acupuncture', *Physical Therapy Reviews,* 16(2), pp. 118-125.

McHeyzer-Williams, M., Okitsu, S., Wang, N. and McHeyzer-Williams, L. (2011) 'Molecular programming of B cell memory', *Nat Rev Immunol,* 12(1), pp. 24-34.

McInnes, I. B. (2017) 'Chapter 26 - Cytokines', in Firestein, G.S., Budd, R.C., Gabriel, S.E., McInnes, I.B. & O'Dell, J.R. (eds.) *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*: Elsevier, pp. 396-407.

McNeil, B. D., Pundir, P., Meeker, S., Han, L., Undem, B. J., Kulka, M. and Dong, X. (2015) 'Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions', *Nature,* 519(7542), pp. 237-41.

Medzhitov, R. and Janeway, C. A., Jr. (2002) 'Decoding the patterns of self and nonself by the innate immune system', *Science,* 296(5566), pp. 298-300.

Meng, X., Al-Attar, Z., Yaseen, F. S., Jenkins, R., Earnshaw, C., Whitaker, P., Peckham, D., French, N. S., Naisbitt, D. J. and Park, B. K. (2017) 'Definition of the Nature and Hapten Threshold of the beta-Lactam Antigen Required for T Cell Activation In Vitro and in Patients', *J Immunol,* 198(11), pp. 4217-4227.

Mennicke, M., Zawodniak, A., Keller, M., Wilkens, L., Yawalkar, N., Stickel, F., Keogh, A., Inderbitzin, D., Candinas, D. and Pichler, W. J. (2009) 'Fulminant liver failure after vancomycin in a sulfasalazine-induced DRESS syndrome: fatal recurrence after liver transplantation', *Am J Transplant,* 9(9), pp. 2197-202.

Miah, M. A., Ahmed, S. S., Chowdhury, S. A., Begum, F. and Rahman, S. H. (2008) 'Fixed drug eruptions due to cotrimoxazole', *Mymensingh Med J,* 17(2 Suppl), pp. S1-5.

Mogensen, T. H. (2009) 'Pathogen recognition and inflammatory signaling in innate immune defenses', *Clinical microbiology reviews,* 22(2), pp. 240-273.

Moller, G. M., de Jong, T. A., van der Kwast, T. H., Overbeek, S. E., Wierenga-Wolf, A. F., Thepen, T. and Hoogsteden, H. C. (1996) 'Immunolocalization of interleukin-4 in eosinophils in the bronchial mucosa of atopic asthmatics', *Am J Respir Cell Mol Biol,* 14(5), pp. 439-43.

Monshi, M. 2013. Characterisation of the cellular basis of beta-lactam induced skin and liver injury. University of Liverpool.

Monshi Manal, M., Faulkner, L., Gibson, A., Jenkins Rosalind, E., Farrell, J., Earnshaw Caroline, J., Alfirevic, A., Cederbrant, K., Daly Ann, K., French, N., Pirmohamed, M., Park, B. K. and Naisbitt Dean, J. (2012) 'Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury', *Hepatology,* 57(2), pp. 727-739.

Monshi, M. M., Faulkner, L., Gibson, A., Jenkins, R. E., Farrell, J., Earnshaw, C. J., Alfirevic, A., Cederbrant, K., Daly, A. K. and French, N. (2013a) 'Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury', (no. 2), pp. 727.

Monshi, M. M., Faulkner, L., Gibson, A., Jenkins, R. E., Farrell, J., Earnshaw, C. J., Alfirevic, A., Cederbrant, K., Daly, A. K., French, N., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2013b) 'Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury', *Hepatology,* 57(2), pp. 727-39.

Monshi, M. M., Faulkner, L., Gibson, A., Jenkins, R. E., Farrell, J., Earnshaw, C. J., Alfirevic, A., Cederbrant, K., Daly, A. K., French, N., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2013c) 'Human leukocyte antigen (HLA)-B*57:01-restricted activation of drug-specific T cells provides the immunological basis for flucloxacillin-induced liver injury', *Hepatology,* 57(2), pp. 727-739.

Morikawa, K., Lange, C. M., Gouttenoire, J., Meylan, E., Brass, V., Penin, F. and Moradpour, D. (2011) 'Nonstructural protein 3-4A: the Swiss army knife of hepatitis C virus', *J Viral Hepat,* 18(5), pp. 305-15.

Mosaad, Y. M. (2015) 'Clinical Role of Human Leukocyte Antigen in Health and Disease', *Scandinavian Journal of Immunology,* 82(4), pp. 283-306.

Mosedale, M., Eaddy, J. S., Trask, O. J., Jr., Holman, N. S., Wolf, K. K., LeCluyse, E., Ware, B. R., Khetani, S. R., Lu, J., Brock, W. J., Roth, S. E. and Watkins, P. B. (2018) 'miR-122 Release in Exosomes Precedes Overt Tolvaptan-Induced Necrosis in a Primary Human Hepatocyte Micropatterned Coculture Model', *Toxicol Sci,* 161(1), pp. 149-158.

Mosedale, M. and Watkins, P. B. (2017) 'Drug-induced liver injury: Advances in mechanistic understanding that will inform risk management', *Clin Pharmacol Ther,* 101(4), pp. 469-480.

Mueller, D. L., Jenkins, M. K. and Schwartz, R. H. (1989) 'Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy', *Annu Rev Immunol,* 7, pp. 445-80.

Murphy, K. (2012) *janeway's IMMUNO BIOLOGY.* 8th edition

edn.

Murphy, K. and Weaver, C. (2017) *Janeway's immunobiology.* New York : Garland Science, Taylor & Francis Group, [2017]

9th ed.

Mustafa, S. S., Ostrov, D. and Yerly, D. (2018) 'Severe Cutaneous Adverse Drug Reactions: Presentation, Risk Factors, and Management', *Current Allergy and Asthma Reports,* 18(4), pp. 26.

Naisbitt, D. J. (2004) 'Drug hypersensitivity reactions in skin: understanding mechanisms and the development of diagnostic and predictive tests', *Toxicology,* 194(3), pp. 179-196.

Naisbitt, D. J., Farrell, J., Gordon, S. F., Maggs, J. L., Burkhart, C., Pichler, W. J., Pirmohamed, M. and Park, B. K. (2002) 'Covalent binding of the nitroso metabolite of sulfamethoxazole leads to toxicity and major histocompatibility complex-restricted antigen presentation', *Mol Pharmacol,* 62(3), pp. 628-37.

Naisbitt, D. J., Farrell, J., Wong, G., Depta, J. P., Dodd, C. C., Hopkins, J. E., Gibney, C. A., Chadwick, D. W., Pichler, W. J., Pirmohamed, M. and Park, B. K. (2003) 'Characterization of drug-specific T cells in lamotrigine hypersensitivity', *J Allergy Clin Immunol,* 111(6), pp. 1393-403.

Naisbitt, D. J., Gordon, S. F., Pirmohamed, M. and Park, B. K. (2000) 'Immunological principles of adverse drug reactions: the initiation and propagation of immune responses elicited by drug treatment', *Drug Saf,* 23(6), pp. 483-507.

Naisbitt, D. J., Hough, S. J., Gill, H. J., Pirmohamed, M., Kitteringham, N. R. and Park, B. K. (1999) 'Cellular disposition of sulphamethoxazole and its metabolites: implications for hypersensitivity', *Br J Pharmacol,* 126(6), pp. 1393-407.

Naisbitt, D. J., O'Neill, P. M., Pirmohamed, M. and Kevin Park, B. (1996a) 'Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune mediated toxicity', *Bioorganic & Medicinal Chemistry Letters,* 6, pp. 1511-1516.

Naisbitt, D. J., Oneill, P. M., Pirmohamed, M. and Park, B. K. 1996b. Synthesis and reactions of nitroso sulphamethoxazole with biological nucleophiles: Implications for immune mediated toxicity.

Nakada, T., Kito, T., Inoue, K., Masuda, S., Inui, K.-i., Matsubara, K., Moriyama, Y., Hisanaga, N., Adachi, Y., Suzuki, M., Yamada, I. and Kusuhara, H. (2014) 'Evaluation of the Potency of Telaprevir and Its Metabolites as Inhibitors of Renal Organic Cation Transporters, a Potential Mechanism for the Elevation of Serum Creatinine', *Drug Metabolism and Pharmacokinetics,* 29(3), pp. 266-271.

Namiki, H. and Kobayashi, T. (2018) 'Long-term, low-dose of clarithromycin as a cause of community-acquired Clostridium difficile infection in a 5-year-old boy', *Oxford Medical Case Reports*, 2018(3), pp. omx106-omx106.

Nassar, A. F., Ogura, H. and Wisnewski, A. V. (2015) 'Impact of recent innovations in the use of mass cytometry in support of drug development', *Drug discovery today*, 20(10), pp. 1169-1175.

Nassif, A., Bensussan, A., Boumsell, L., Deniaud, A., Moslehi, H., Wolkenstein, P., Bagot, M. and Roujeau, J.-C. (2004a) 'Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells', *The Journal Of Allergy And Clinical Immunology*, 114(5), pp. 1209-1215.

Nassif, A., Bensussan, A., Boumsell, L., Deniaud, A., Moslehi, H., Wolkenstein, P., Bagot, M. and Roujeau, J. C. (2004b) 'Toxic epidermal necrolysis: effector cells are drug-specific cytotoxic T cells', *J Allergy Clin Immunol*, 114(5), pp. 1209-15.

Nebeker, J. R., Barach, P. and Samore, M. H. (2004) 'Clarifying adverse drug events: a clinician's guide to terminology, documentation, and reporting', *Ann Intern Med*, 140(10), pp. 795-801.

Neefjes, J., Jongsma, M. L. M., Paul, P. and Bakke, O. (2011) 'Towards a systems understanding of MHC class I and MHC class II antigen presentation', *Nature Reviews Immunology*, 11, pp. 823.

Neitzel, H. (1986) 'A routine method for the establishment of permanent growing lymphoblastoid cell lines', *Hum Genet*, 73(4), pp. 320-6.

Neurath, M. F. and Finotto, S. (2016) 'IL-9 signaling as key driver of chronic inflammation in mucosal immunity', *Cytokine & Growth Factor Reviews*, 29, pp. 93-99.

Ngoenkam, J., Schamel, W. W. and Pongcharoen, S. (2017) 'Selected signalling proteins recruited to the T-cell receptor–CD3 complex', *Immunology*, 153(1), pp. 42-50.

Nicole Cutler, L. A. (2013) 'The Dark Side of Telaprevir', *Hepatitis central*.

Nika, K., Soldani, C., Salek, M., Paster, W., Gray, A., Etzensperger, R., Fugger, L., Polzella, P., Cerundolo, V., Dushek, O., Hofer, T., Viola, A. and Acuto, O. (2010) 'Constitutively active Lck kinase in T cells drives antigen receptor signal transduction', *Immunity*, 32(6), pp. 766-77.

Norcross, M. A., Luo, S., Lu, L., Boyne, M. T., Gomarteli, M., Rennels, A. D., Woodcock, J., Margulies, D. H., McMurtrey, C., Vernon, S., Hildebrand, W. H. and Buchli, R. (2012) 'Abacavir induces loading of novel self-peptides into HLA-B*57: 01: an autoimmune model for HLA-associated drug hypersensitivity', *Aids*, 26(11), pp. F21-9.

Nurieva, R. I., Chung, Y., Martinez, G. J., Yang, X. O., Tanaka, S., Matskevitch, T. D., Wang, Y. H. and Dong, C. (2009) 'Bcl6 mediates the development of T follicular helper cells', *Science*, 325(5943), pp. 1001-5.

Oleinika, K., Rosser, E. C., Matei, D. E., Nistala, K., Bosma, A., Drozdov, I. and Mauri, C. (2018) 'CD1d-dependent immune suppression mediated by regulatory B cells through modulations of iNKT cells', *Nature Communications*, 9(1), pp. 684.

Organization, W. H. (2017) 'Global Hepatitis C report', *World Health Organization*.

Ostrov, D. A., Grant, B. J., Pompeu, Y. A., Sidney, J., Harndahl, M., Southwood, S., Oseroff, C., Lu, S., Jakoncic, J., de Oliveira, C. A., Yang, L., Mei, H., Shi, L., Shabanowitz, J., English, A. M., Wriston, A., Lucas, A., Phillips, E., Mallal, S., Grey, H. M., Sette, A., Hunt, D. F., Buus, S. and Peters, B. (2012) 'Drug hypersensitivity caused by alteration of the MHC-presented self-peptide repertoire', *Proc Natl Acad Sci U S A*, 109(25), pp. 9959-64.

Padovan, E., Mauri-Hellweg, D., Pichler, W. J. and Weltzien, H. U. (1996) 'T cell recognition of penicillin G: structural features determining antigenic specificity', *Eur J Immunol,* 26, pp. 42-8.

Page, R. C. L. (2011) 'Chapter 43 - Miscellaneous hormones', in Aronson, J.K. (ed.) *Side Effects of Drugs Annual*: Elsevier, pp. 909-919.

Pai, M. P. and Bertino, J. S. (2015) '54 - Tables of Anti-infective Agent Pharmacology', in Bennett, J.E., Dolin, R. & Blaser, M.J. (eds.) *Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases (Eighth Edition)*. Philadelphia: Content Repository Only!, pp. 631-707.

Pal, A., Sen, S., Das, S., Biswas, A. and Tripathi, S. K. (2014) 'A Case of Self-treatment Induced Recurrent Fixed Drug Eruptions Associated with the Use of Different Fixed Dose Combinations of Fluoroquinolone-Nitroimidazole', *Iranian Journal of Medical Sciences,* 39(6), pp. 584-588.

Parham, P. and Janeway, C. A. (2009) *The immune system.* London : Garland Science, 2009.

3rd ed.

Park, B. K., Boobis, A., Clarke, S., Goldring, C. E., Jones, D., Kenna, J. G., Lambert, C., Laverty, H. G., Naisbitt, D. J., Nelson, S., Nicoll-Griffith, D. A., Obach, R. S., Routledge, P., Smith, D. A., Tweedie, D. J., Vermeulen, N., Williams, D. P., Wilson, I. D. and Baillie, T. A. (2011a) 'Managing the challenge of chemically reactive metabolites in drug development', *Nat Rev Drug Discov,* 10(4), pp. 292-306.

Park, B. K., Laverty, H., Srivastava, A., Antoine, D. J., Naisbitt, D. and Williams, D. P. (2011b) 'Drug bioactivation and protein adduct formation in the pathogenesis of drug-induced toxicity', *Chemico-Biological Interactions,* 192(1), pp. 30-36.

Park, B. K., Pirmohamed, M. and Kitteringham, N. R. (1998) 'Role of drug disposition in drug hypersensitivity: a chemical, molecular, and clinical perspective', *Chemical Research In Toxicology,* 11(9), pp. 969-988.

Patton, K. and Borshoff, D. C. (2018) 'Adverse drug reactions', *Anaesthesia,* 73(S1), pp. 76-84.

Pavlos, R., Mallal, S., Ostrov, D., Buus, S., Metushi, I., Peters, B. and Phillips, E. (2015) 'T cell-mediated hypersensitivity reactions to drugs', *Annu Rev Med,* 66, pp. 439-54.

Pavlos, R., Mallal, S. and Phillips, E. (2012) 'HLA and pharmacogenetics of drug hypersensitivity', *Pharmacogenomics,* 13(11), pp. 1285-306.

Pichler, W. J. (2002) 'Pharmacological interaction of drugs with antigen-specific immune receptors: The p-i concept', *Current Opinion in Allergy and Clinical Immunology,* 2(4), pp. 301-305.

Pichler, W. J. (2003) 'Delayed drug hypersensitivity reactions', *Ann Intern Med,* 139(8), pp. 683-93.

Pichler, W. J. (2005) 'Direct T-cell stimulations by drugs--bypassing the innate immune system', *Toxicology,* 209(2), pp. 95-100.

Pichler, W. J. (2007) ' Drug Hypersensitivity Reactions:Classification and Relationship to T-Cell Activation', pp. 168-189.

Pichler, W. J. (2008) 'The p-i Concept: Pharmacological Interaction of Drugs With Immune Receptors', *World Allergy Organ J,* 1(6), pp. 96-102.

Pichler, W. J. (2013) 'Consequences of drug binding to immune receptors: Immune stimulation following pharmacological interaction with immune receptors (T-cell receptor for antigen or human leukocyte antigen) with altered peptide-human leukocyte antigen or peptide', *Dermatologica Sinica,* 31(4), pp. 181-190.

Pichler, W. J., Adam, J., Daubner, B., Gentinetta, T., Keller, M. and Yerly, D. (2010) 'Drug hypersensitivity reactions: pathomechanism and clinical symptoms', *Med Clin North Am,* 94(4), pp. 645-64, xv.

Pichler, W. J., Adam, J., Watkins, S., Wuillemin, N., Yun, J. and Yerly, D. (2015) 'Drug Hypersensitivity: How Drugs Stimulate T Cells via Pharmacological Interaction with Immune Receptors', *Int Arch Allergy Immunol,* 168(1), pp. 13-24.

Pichler, W. J., Beeler, A., Keller, M., Lerch, M., Posadas, S., Schmid, D., Spanou, Z., Zawodniak, A. and Gerber, B. (2006) 'Pharmacological interaction of drugs with immune receptors: The p-i concept', *Allergology International,* 55(1), pp. 17-25.

Pichler, W. J., Naisbitt, D. J. and Park, B. K. (2011) 'Immune pathomechanism of drug hypersensitivity reactions', *J Allergy Clin Immunol,* 127(3 Suppl), pp. S74-81.

Pirmohamed, M., Breckenridge, A. M., Kitteringham, N. R. and Park, B. K. (1998) 'Adverse drug reactions', *BMJ,* 316(7140), pp. 1295.

Pirmohamed, M., James, S., Meakin, S., Green, C., Scott, A. K., Walley, T. J., Farrar, K., Park, B. K. and Breckenridge, A. M. (2004) 'Adverse drug reactions as cause of admission to hospital: prospective analysis of 18 820 patients', *Bmj,* 329(7456), pp. 15-9.

Pirmohamed, M., Naisbitt, D. J., Gordon, F. and Park, B. K. (2002) 'The danger hypothesis—potential role in idiosyncratic drug reactions', *Toxicology,* 181-182, pp. 55-63.

Pirmohamed, M., Ostrov, D. A. and Park, B. K. (2015) 'New genetic findings lead the way to a better understanding of fundamental mechanisms of drug hypersensitivity', *J Allergy Clin Immunol,* 136(2), pp. 236-44.

Pirmohamed, M. and Park, K. (1997) 'Mechanism of clozapine-induced agranulocytosis : current status of research and implications for drug development', *CNS Drugs,* 7(2), pp. 139-58.

Pirmohamed, M., Williams, D., Madden, S., Templeton, E. and Park, B. K. (1995) 'Metabolism and bioactivation of clozapine by human liver in vitro', *Journal of Pharmacology and Experimental Therapeutics,* 272(3), pp. 984-990.

Posadas, S. J., Padial, A., Torres, M. J., Mayorga, C., Leyva, L., Sanchez, E., Alvarez, J., Romano, A., Juarez, C. and Blanca, M. (2002) 'Delayed reactions to drugs show levels of perforin, granzyme B, and Fas-L to be related to disease severity', *Journal of allergy and clinical immunology,* 109(1), pp. 155-161.

Posadas, S. J. and Pichler, W. J. (2007) 'Delayed drug hypersensitivity reactions - new concepts', *Clin Exp Allergy,* 37(7), pp. 989-99.

PubChem (2018a) 'National Center for Biotechnology Information. Compound Database;'.

Pubchem (2018b) ' PubChem Compound Database/ dapsone ', *National Center for Biotechnology Information.*

PubChem (2018c) 'PubChem Compound Database/ nitroso dapsone ', *National Center for Biotechnology Information.*

Punt, J. (2013) 'Chapter 4 - Adaptive Immunity: T Cells and Cytokines', in Prendergast, G.C. & Jaffee, E.M. (eds.) *Cancer Immunotherapy (Second Edition)*. San Diego: Academic Press, pp. 41-53.

Raeber Miro, E., Zurbuchen, Y., Impellizzieri, D. and Boyman, O. (2018) 'The role of cytokines in T-cell memory in health and disease', *Immunological Reviews,* 283(1), pp. 176-193.

Rajan, T. V. (2003) 'The Gell-Coombs classification of hypersensitivity reactions: a re-interpretation', *Trends Immunol,* 24(7), pp. 376-9.

Rao, P. N. and Lakshmi, T. S. (2001) 'Increase in the incidence of dapsone hypersensitivity syndrome--an appraisal', *Lepr Rev,* 72(1), pp. 57-62.

Rawlins, M. and Thompson, J. 1991. Mechanisms of adverse drug reactions. En: Davies D., editors. Textbook of adverse drug reactions. 4 th. Oxford: Oxford University Press.

Rawlins MD and JW, T. (1991) *Mechanisms of adverse drug reactions. In: Davies DM, editor. Textbook of adverse drug reactions.* . p. 18–45.

Rebuli, M. E., Pawlak, E. A., Walsh, D., Martin, E. M. and Jaspers, I. (2018) 'Distinguishing Human Peripheral Blood NK Cells from CD56(dim)CD16(dim)CD69(+)CD103(+) Resident Nasal Mucosal Lavage Fluid Cells', *Sci Rep,* 8(1), pp. 3394.

Redwood, A. J., Pavlos, R. K., White, K. D. and Phillips, E. J. (2018) 'HLAs: Key regulators of T-cell-mediated drug hypersensitivity', *Hla,* 91(1), pp. 3-16.

Renard, D. and Rosselet, A. (2017) 'Drug-induced hemolytic anemia: Pharmacological aspects', *Transfusion Clinique et Biologique,* 24(3), pp. 110-114.

Rhoades, R. and Bell, D. R. (2009) *Medical physiology : principles for clinical medicine.* Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins, ò013.

4th International ed.

Rhoades, R. and Bell, D. R. (2013) *Medical physiology. [electronic book] : principles for clinical medicine. Online access with subscription: LWW Health Library (Integrated Basic Science Collection)*: Philadelphia : Wolters Kluwer Health/Lippincott Williams & Wilkins, 2013.

4th ed.

Roos, D. (1998) 'Neutrophils', in Delves, P.J. (ed.) *Encyclopedia of Immunology (Second Edition)*. Oxford: Elsevier, pp. 1854-1858.

Rothenberg, M. E. (1998) 'Eosinophilia', *N Engl J Med,* 338(22), pp. 1592-600.

Rothenberg, M. E. and Hogan, S. P. (2006) 'The eosinophil', *Annu Rev Immunol,* 24, pp. 147-74.

Roughead, E. E. (2002) 'The nature and extent of drug-related hospitalisations in Australia', *Journal of Quality in Clinical Practice,* 19(1), pp. 19-22.

Roujeau, J., Mockenhaupt, M., Tahan, S. R. and et al. (2013a) 'Telaprevir-related dermatitis', *JAMA Dermatology,* 149(2), pp. 152-158.

Roujeau, J. C. (2005) 'Clinical heterogeneity of drug hypersensitivity', *Toxicology,* 209(2), pp. 123-9.

Roujeau, J. C., Mockenhaupt, M., Tahan, S. R., Henshaw, J., Martin, E. C., Harding, M., van Baelen, B., Bengtsson, L., Singhal, P., Kauffman, R. S. and Stern, R. S. (2013b) 'Telaprevir-related dermatitis', *JAMA Dermatol,* 149(2), pp. 152-8.

Roychowdhury, S., Vyas, P. M. and Svensson, C. K. (2007) 'Formation and uptake of arylhydroxylamine-haptenated proteins in human dendritic cells', *Drug metabolism and disposition: the biological fate of chemicals,* 35(4), pp. 676-681.

Sachs, B., Erdmann, S., Al-Masaoudi, T. and Merk, H. F. (2001) 'In vitro drug allergy detection system incorporating human liver microsomes in chlorazepate-induced skin rash: drug-specific proliferation associated with interleukin-5 secretion', *The British Journal Of Dermatology,* 144(2), pp. 316-320.

Sahay, M. and Sahay, R. (2014) 'Hyponatremia: A practical approach', *Indian Journal of Endocrinology and Metabolism,* 18(6), pp. 760-771.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M. and Toda, M. (1995) 'Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases', *J Immunol,* 155(3), pp. 1151-64.

Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A. (1999) 'Two subsets of memory T lymphocytes with distinct homing potentials and effector functions', *Nature,* 401(6754), pp. 708-12.

Sanchez-Politta, S., Angelillo-Scherrer, A., Masouyé, I. and Borradori, L. (2006) 'Widespread skin necrosis associated with unfractionated heparin therapy in a patient under chronic coumarin anticoagulation', *Journal of the European Academy of Dermatology and Venereology,* 20(3), pp. 327-330.

Sanderson, J. P., Naisbitt, D. J., Farrell, J., Ashby, C. A., Tucker, M. J., Rieder, M. J., Pirmohamed, M., Clarke, S. E. and Park, B. K. (2007) 'Sulfamethoxazole and its metabolite nitroso sulfamethoxazole stimulate dendritic cell costimulatory signaling', *J Immunol,* 178(9), pp. 5533-42.

Schaerli, P., Willimann, K., Lang, A. B., Lipp, M., Loetscher, P. and Moser, B. (2000) 'CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function', *J Exp Med,* 192(11), pp. 1553-62.

Schilham, M. W., Fung-Leung, W. P., Rahemtulla, A., Kuendig, T., Zhang, L., Potter, J., Miller, R. G., Hengartner, H. and Mak, T. W. (1993) 'Alloreactive cytotoxic T cells can develop and function in mice lacking both CD4 and CD8', *Eur J Immunol,* 23(6), pp. 1299-304.

Schlapbach, C., Zawodniak, A., Irla, N., Adam, J., Hunger, R. E., Yerly, D., Pichler, W. J. and Yawalkar, N. (2011) 'NKp46+ cells express granulysin in multiple cutaneous adverse drug reactions', *Allergy,* 66(11), pp. 1469-76.

Schmid, D. A., Depta, J. P. H., Lüthi, M. and Pichler, W. J. (2006) 'Transfection of Drug-Specific T-Cell Receptors into Hybridoma Cells: Tools to Monitor Drug Interaction with T-Cell Receptors and Evaluate Cross-Reactivity to Related Compounds', *Molecular Pharmacology,* 70(1), pp. 356.

Schmidt, A., Oberle, N. and Krammer, P. (2012) 'Molecular Mechanisms of Treg-Mediated T Cell Suppression', *Frontiers in Immunology,* 3(51).

Schnyder, B., Burkhart, C., Schnyder-Frutig, K., von Greyerz, S., Naisbitt, D. J., Pirmohamed, M., Park, B. K. and Pichler, W. J. (2000) 'Recognition of sulfamethoxazole and its reactive metabolites by drug-specific CD4+ T cells from allergic individuals', *Journal Of Immunology (Baltimore, Md.: 1950),* 164(12), pp. 6647-6654.

Schnyder, B., Mauri-Hellweg, D., Zanni, M., Bettens, F. and Pichler, W. J. (1997) 'Direct, MHC-dependent presentation of the drug sulfamethoxazole to human alphabeta T cell clones', *J Clin Invest,* 100(1), pp. 136-41.

Schnyder, B. and Pichler, W. J. (2009) 'Mechanisms of Drug-Induced Allergy', *Mayo Clinic Proceedings,* 84(3), pp. 268-272.

Schroeder, H. W. and Cavacini, L. (2010) 'Structure and Function of Immunoglobulins', *The Journal of allergy and clinical immunology,* 125(2 0 2), pp. S41-S52.

Schwarz, K. and Bartram, C. R. (1996) 'V(D)J Recombination Pathology', in Dixon, F.J. (ed.) *Advances in Immunology*: Academic Press, pp. 285-326.

Seishima, M., Yamanaka, S., Fujisawa, T., Tohyama, M. and Hashimoto, K. (2006) 'Reactivation of human herpesvirus (HHV) family members other than HHV-6 in drug-induced hypersensitivity syndrome', *Br J Dermatol,* 155(2), pp. 344-9.

Semrock (2018) 'Flow Cytometry - Semrock [Online]. '.

Setton, J., Bindra, R. S. and Powell, S. N. (2016) 'Chapter 9 - DNA double-strand repair by nonhomologous end joining and its clinical relevance', in Kelley, M.R. & Fishel, M.L. (eds.) *DNA Repair in Cancer Therapy (Second Edition)*. Boston: Academic Press, pp. 277-302.

Shah, B., Burg, N. and Pillinger, M. H. (2017) 'Chapter 11 - Neutrophils', in Firestein, G.S., Budd, R.C., Gabriel, S.E., McInnes, I.B. & O'Dell, J.R. (eds.) *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*: Elsevier, pp. 169-188.e3.

Shah, S., Shah, H., Khaskheli, M. N. and Akhtar, J. (2005) 'Adverse drug reactions: clinical assessment of drug induced disease', *J Ayub Med Coll Abbottabad,* 17(1), pp. 89-91.

Shear, N. H. and Spielberg, S. P. (1988) 'Anticonvulsant hypersensitivity syndrome. In vitro assessment of risk', *J Clin Invest,* 82(6), pp. 1826-32.

Shoaf, S. E., Bricmont, P. and Mallikaarjun, S. (2012) 'Absolute bioavailability of tolvaptan and determination of minimally effective concentrations in healthy subjects', *Int J Clin Pharmacol Ther,* 50(2), pp. 150-6.

Shulman, S. T. (2017) 'Clemens von Pirquet: A Remarkable Life and Career', *J Pediatric Infect Dis Soc,* 6(4), pp. 376-379.

Shuttleworth, S., Townsend, P., Silva, F., Cecil, A., Hill, T., Tomassi, C., Rogers, H. and Harrison, R. (2011) 'Progress in the Development of Small Molecule Therapeutics Targeting Th17 Cell Function for the Treatment of Immune-Inflammatory Diseases', in Lawton, G. & Witty, D.R. (eds.) *Progress in Medicinal Chemistry*: Elsevier, pp. 109-133.

Singer, J. B., Lewitzky, S., Leroy, E., Yang, F., Zhao, X., Klickstein, L., Wright, T. M., Meyer, J. and Paulding, C. A. (2010) 'A genome-wide study identifies HLA alleles associated with lumiracoxib-related liver injury', *Nat Genet,* 42(8), pp. 711-4.

Singh, B., Schwartz, J. A., Sandrock, C., Bellemore, S. M. and Nikoopour, E. (2013) 'Modulation of autoimmune diseases by interleukin (IL)-17 producing regulatory T helper (Th17) cells', (no. 5), pp. 591.

Smith-Garvin, J. E., Koretzky, G. A. and Jordan, M. S. (2009) 'T cell activation', *Annu Rev Immunol,* 27, pp. 591-619.

Smith, L. S., Nelson, M., Naik, S. and Woten, J. (2011) 'Telaprevir: an NS3/4A protease inhibitor for the treatment of chronic hepatitis C', *Ann Pharmacother,* 45(5), pp. 639-48.

Souza-Fonseca-Guimaraes, F., Parlato, M., Philippart, F., Misset, B., Cavaillon, J. M. and Adib-Conquy, M. (2012) 'Toll-like receptors expression and interferon-gamma production by NK cells in human sepsis', *Crit Care,* 16(5), pp. R206.

Spraggs, C. F., Budde, L. R., Briley, L. P., Bing, N., Cox, C. J., King, K. S., Whittaker, J. C., Mooser, V. E., Preston, A. J., Stein, S. H. and Cardon, L. R. (2011) 'HLA-DQA1*02:01 is a major risk factor for lapatinib-induced hepatotoxicity in women with advanced breast cancer', *J Clin Oncol,* 29(6), pp. 667-73.

Stark, M. A., Huo, Y., Burcin, T. L., Morris, M. A., Olson, T. S. and Ley, K. (2005) 'Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17', *Immunity,* 22(3), pp. 285-94.

Stauss, H. and Xue, S.-A. (2014) *T-cell receptor capable of recognising an antigen from cytomegalovirus*. [Online]. Available at: https://liverpool.idm.oclc.org/login?url=https://search.ebscohost.com/login.aspx?direct=true&db=edspgr&AN=edspgr.08722048&site=eds-live&scope=site (Accessed: 20140513).

Steinman, R. M. (1991) 'The dendritic cell system and its role in immunogenicity', *Annu Rev Immunol,* 9, pp. 271-96.

Steinman, R. M. and Nussenzweig, M. C. (2002) 'Avoiding horror autotoxicus: the importance of dendritic cells in peripheral T cell tolerance', *Proc Natl Acad Sci U S A,* 99(1), pp. 351-8.

Storkus, W. J., Howell, D. N., Salter, R. D., Dawson, J. R. and Cresswell, P. (1987) 'NK susceptibility varies inversely with target cell class I HLA antigen expression', *The Journal of Immunology,* 138(6), pp. 1657.

Suda, G., Yamamoto, Y., Nagasaka, A., Furuya, K., Kudo, M., Chuganji, Y., Tsukuda, Y., Tsunematsu, S., Sato, F., Terasita, K., Nakai, M., Horimoto, H., Sho, T., Natsuizaka,

M., Ogawa, K., Ohnishi, S., Chuma, M., Fujita, Y., Abe, R., Taniguchi, M., Nakagawa, M., Asahina, Y. and Sakamoto, N. (2015) 'Serum granulysin levels as a predictor of serious telaprevir-induced dermatological reactions', *Hepatol Res,* 45(8), pp. 837-45.

Sullivan, A., Wang, E., Farrell, J., Whitaker, P., Faulkner, L., Peckham, D., Park, B. K. and Naisbitt, D. J. (2018) 'beta-Lactam hypersensitivity involves expansion of circulating and skin-resident TH22 cells', *J Allergy Clin Immunol,* 141(1), pp. 235-249.e8.

Sultana, J., Cutroneo, P. and Trifirò, G. (2013) 'Clinical and economic burden of adverse drug reactions', *Journal of Pharmacology & Pharmacotherapeutics,* 4(Suppl1), pp. S73-S77.

Suresh, R. and Mosser, D. M. (2013) 'Pattern recognition receptors in innate immunity, host defense, and immunopathology', *Advances in physiology education,* 37(4), pp. 284-291.

Surh, C. D. and Sprent, J. (2008) 'Homeostasis of naive and memory T cells', *Immunity,* 29(6), pp. 848-62.

Sutton, C. E., Lalor, S. J., Sweeney, C. M., Brereton, C. F., Lavelle, E. C. and Mills, K. H. G. (2009) 'Interleukin-1 and IL-23 Induce Innate IL-17 Production from γδ T Cells, Amplifying Th17 Responses and Autoimmunity', *Immunity,* 31(2), pp. 331-341.

Swainson, L., de Barros, S. C., Craveiro, M., Zimmermann, V. S. and Taylor, N. (2013) '8 - T-cell development', in Rich, R.R., Fleisher, T.A., Shearer, W.T., Schroeder, H.W., Frew, A.J. & Weyand, C.M. (eds.) *Clinical Immunology (Fourth Edition)*. London: Content Repository Only!, pp. 102-107.

Tacke, F., Alvarez, D., Kaplan, T. J., Jakubzick, C., Spanbroek, R., Llodra, J., Garin, A., Liu, J., Mack, M., van Rooijen, N., Lira, S. A., Habenicht, A. J. and Randolph, G. J. (2007) 'Monocyte subsets differentially employ CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques', *J Clin Invest,* 117(1), pp. 185-94.

Tailor, A., Faulkner, L., Naisbitt, D. J. and Park, B. K. (2015) 'The chemical, genetic and immunological basis of idiosyncratic drug–induced liver injury', *Human & Experimental Toxicology,* 34(12), pp. 1310-1317.

Teh, L. K., Selvaraj, M., Bannur, Z., Ismail, M. I., Rafia, H., Law, W. C., Sapuan, S., Puvanarajah, S., Ali, P. S. and Salleh, M. Z. (2016) 'Coupling Genotyping and Computational Modeling in Prediction of Anti-epileptic Drugs that cause Stevens Johnson Syndrome and Toxic Epidermal Necrolysis for Carrier of HLA-B*15:02', *J Pharm Pharm Sci,* 19(1), pp. 147-60.

Tempark, T., Satapornpong, P., Rerknimitr, P., Nakkam, N., Saksit, N., Wattanakrai, P., Jantararoungtong, T., Koomdee, N., Mahakkanukrauh, A., Tassaneeyakul, W., Suttisai, S., Pratoomwun, J., Klaewsongkram, J., Rerkpattanapipat, T. and Sukasem, C. (2017) 'Dapsone-induced severe cutaneous adverse drug reactions are strongly linked with HLA-B*13: 01 allele in the Thai population', *Pharmacogenet Genomics,* 27(12), pp. 429-437.

Tesmer, L. A., Lundy, S. K., Sarkar, S. and Fox, D. A. (2008) 'Th17 cells in human disease', *Immunol Rev,* 223, pp. 87-113.

Thornton, A. M. and Shevach, E. M. (1998) 'CD4⁺CD25⁺ Immunoregulatory T Cells Suppress Polyclonal T Cell Activation In Vitro by Inhibiting Interleukin 2 Production', *The Journal of Experimental Medicine,* 188(2), pp. 287.

Toews, G. B. (2009) 'Chapter 11 - Macrophages', in Barnes, P.J., Drazen, J.M., Rennard, S.I. & Thomson, N.C. (eds.) *Asthma and COPD (Second Edition)*. Oxford: Academic Press, pp. 133-143.

Tomasek, J. J., Papka, R. E., McNeill, D. L., Burden, H. W. and Papka, R. E. (2011) *Anatomy: Embryology - Gross Anatomy - Neuroanatomy - Microanatomy*. Springer New York.

Tonegawa, S. (1983) 'Somatic generation of antibody diversity', *Nature*, 302(5909), pp. 575-81.

Torres, M. A. and Moraes, M. E. H. (2011) 'Nomenclature for factors of the HLA system', *Einstein (São Paulo)*, 9, pp. 249-251.

Torres, V. E. (2018) 'Pro: Tolvaptan delays the progression of autosomal dominant polycystic kidney disease', *Nephrol Dial Transplant*.

Torres, V. E., Chapman, A. B., Devuyst, O., Gansevoort, R. T., Grantham, J. J., Higashihara, E., Perrone, R. D., Krasa, H. B., Ouyang, J. and Czerwiec, F. S. (2012) 'Tolvaptan in patients with autosomal dominant polycystic kidney disease', *N Engl J Med*, 367(25), pp. 2407-18.

Uetrecht, J. and Naisbitt, D. J. (2013) 'Idiosyncratic Adverse Drug Reactions: Current Concepts', *Pharmacological Reviews*, 65(2), pp. 779-808.

Um, S. J., Lee, S. K., Kim, Y. H., Kim, K. H., Son, C. H., Roh, M. S. and Lee, M. K. (2010) 'Clinical features of drug-induced hypersensitivity syndrome in 38 patients', *J Investig Allergol Clin Immunol*, 20(7), pp. 556-62.

Usui, T., Faulkner, L., Farrell, J., French, N. S., Alfirevic, A., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2018) 'Application of in Vitro T Cell Assay Using Human Leukocyte Antigen-Typed Healthy Donors for the Assessment of Drug Immunogenicity', *Chemical Research in Toxicology*, 31(3), pp. 165-167.

Usui, T., Meng, X., Saide, K., Farrell, J., Thomson, P., Whitaker, P., Watson, J., French, N. S., Kevin Park, B. and Naisbitt, D. J. (2017a) 'From the Cover: Characterization of Isoniazid-Specific T-Cell Clones in Patients with anti-Tuberculosis Drug-Related Liver and Skin Injury', *Toxicol Sci*, 155(2), pp. 420-431.

Usui, T., Meng, X., Saide, K., Farrell, J., Thomson, P., Whitaker, P., Watson, J., French, N. S., Kevin Park, B. and Naisbitt, D. J. (2017b) 'From the Cover: Characterization of Isoniazid-Specific T-Cell Clones in Patients with anti-Tuberculosis Drug-Related Liver and Skin Injury', *Toxicological Sciences: An Official Journal Of The Society Of Toxicology*, 155(2), pp. 420-431.

Usui, T., Whitaker, P., Meng, X., Watson, J., Antoine, D. J., French, N. S., Park, B. K. and Naisbitt, D. J. (2016) 'Detection of Drug-Responsive T-Lymphocytes in a Case of Fatal Antituberculosis Drug-Related Liver Injury', *Chemical Research in Toxicology*, 29(11), pp. 1793-1795.

Van Acker, H. H., Capsomidis, A., Smits, E. L. and Van Tendeloo, V. F. (2017) 'CD56 in the Immune System: More Than a Marker for Cytotoxicity?', *Frontiers in immunology*, 8, pp. 892-892.

Van Den Driessche, G. and Fourches, D. (2017) 'Adverse drug reactions triggered by the common HLA-B*57:01 variant: a molecular docking study', *J Cheminform*, 9, pp. 13.

van der Merwe, P. and Dushek, O. (2011) *Mechanisms of T cell receptor triggering*.

Varma, R. (2008) *TCR Triggering by the pMHC Complex: Valency, Affinity, and Dynamics*.

Venkatesan, K. (1997) 'Pharmacokinetics and drug interactions of newer anti -leprosy drugs', *Indian Journal of Dermatology, Venereology and Leprology, Vol 63, Iss 3, Pp 148-152 (1997)*, (3), pp. 148.

Verna, E. C. and Brown, R. S., Jr. (2006a) 'Hepatitis C Virus and Liver Transplantation', *Clinics in Liver Disease*, 10(4), pp. 919-940.

Verna, E. C. and Brown, R. S., Jr. (2006b) 'Hepatitis C virus and liver transplantation', *Clin Liver Dis*, 10(4), pp. 919-40.

Vertex. (2013) 'INCIVEK [Online]. Vertex Pharmaceuticals Incorporated.': Vertex Pharmaceuticals (Canada) Incorporated.

Vinod, K. V., Arun, K. and Dutta, T. K. (2013) 'Dapsone hypersensitivity syndrome: A rare life threatening complication of dapsone therapy', *Journal of Pharmacology & Pharmacotherapeutics,* 4(2), pp. 158-160.

Vitezica, Z. G., Milpied, B., Lonjou, C., Borot, N., Ledger, T. N., Lefebvre, A. and Hovnanian, A. (2008) 'HLA-DRB1*01 associated with cutaneous hypersensitivity induced by nevirapine and efavirenz', *AIDS,* 22(4), pp. 540-541 10.1097/QAD.0b013e3282f37812.

Vivier, E., Raulet, D. H., Moretta, A., Caligiuri, M. A., Zitvogel, L., Lanier, L. L., Yokoyama, W. M. and Ugolini, S. (2011) 'Innate or adaptive immunity? The example of natural killer cells', *Science,* 331(6013), pp. 44-9.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T. and Ugolini, S. (2008) 'Functions of natural killer cells', *Nat Immunol,* 9(5), pp. 503-10.

von Greyerz, S., Bültemann, G., Schnyder, K., Burkhart, C., Lotti, B., Hari, Y. and Pichler, W. J. (2001a) 'Degeneracy and additional alloreactivity of drug-specific human alpha beta(+) T cell clones', *International Immunology,* 13(7), pp. 877-885.

von Greyerz, S., Bültemann, G., Schnyder, K., Burkhart, C., Lotti, B., Hari, Y. and Pichler, W. J. (2001b) 'Degeneracy and additional alloreactivity of drug-specific human αβ+ T cell clones', *International Immunology,* 13(7), pp. 877-885.

von Knebel Doeberitz, M., Bornkamm, G. W. and zur Hausen, H. (1983) 'Establishment of spontaneously outgrowing lymphoblastoid cell lines with Cyclosporin A', *Med Microbiol Immunol,* 172(2), pp. 87-99.

Vos, Q., Lees, A., Wu, Z. Q., Snapper, C. M. and Mond, J. J. (2000) 'B-cell activation by T-cell-independent type 2 antigens as an integral part of the humoral immune response to pathogenic microorganisms', *Immunol Rev,* 176, pp. 154-70.

Vyas, P. M., Roychowdhury, S., Khan, F. D., Prisinzano, T. E., Lamba, J., Schuetz, E. G., Blaisdell, J., Goldstein, J. A., Munson, K. L., Hines, R. N. and Svensson, C. K. (2006a) 'Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes: I. Expression and role of cytochromes P450', *J Pharmacol Exp Ther,* 319(1), pp. 488-96.

Vyas, P. M., Roychowdhury, S., Khan, F. D., Prisinzano, T. E., Lamba, J. K., Schuetz, E. G., Blaisdell, J., Goldstein, J. A., Munson, K. L., Hines, R. N. and Svensson, C. (2006b) 'Enzyme-mediated protein haptenation of dapsone and sulfamethoxaozle in human keratinocytes - 1. Expression and role of cytochromes P450', *J Pharmacol Exp Ther*.

Vyas, P. M., Roychowdhury, S., Koukouritaki, S. B., Hines, R. N., Krueger, S. K., Williams, D. E., Nauseef, W. M. and Svensson, C. K. (2006c) 'Enzyme-mediated protein haptenation of dapsone and sulfamethoxazole in human keratinocytes - 2. Expression and role of flavin-containing monooxygeanses and peroxidases', *J Pharmacol Exp Ther*.

Walker, S. L., Withington, S. G. and Lockwood, D. N. J. (2014) '41 - Leprosy A2 - Farrar, Jeremy', in Hotez, P.J., Junghanss, T., Kang, G., Lalloo, D. & White, N.J. (eds.) *Manson's Tropical Infectious Diseases (Twenty-Third Edition)*. London: W.B. Saunders, pp. 506-518.e1.

Walsh, S. A. and Creamer, D. (2011) 'Drug reaction with eosinophilia and systemic symptoms (DRESS): a clinical update and review of current thinking', *Clinical and Experimental Dermatology,* 36(1), pp. 6-11.

Wan, W. L., Wu, J. B., Lei, F., Li, X. L., Hai, L. and Wu, Y. (2012) 'Synthesis of the major metabolites of Tolvaptan', *Chinese Chemical Letters,* 23(12), pp. 1343-1346.

Warkentin, T. E. (2003) 'Heparin-induced thrombocytopenia: pathogenesis and management', *Br J Haematol,* 121(4), pp. 535-55.

Warrington, R. (2012) 'Drug allergy: causes and desensitization', *Human vaccines & immunotherapeutics,* 8(10), pp. 1513-1524.

Warrington, R., Silviu-Dan, F. and Wong, T. (2018) 'Drug allergy', *Allergy, Asthma & Clinical Immunology,* 14(2), pp. 60.

Watanabe, H., Watanabe, Y., Tashiro, Y., Mushiroda, T., Ozeki, T., Hashizume, H., Sueki, H., Yamamoto, T., Utsunomiya-Tate, N., Gouda, H. and Kusakabe, Y. (2017) 'A docking model of dapsone bound to HLA-B*13:01 explains the risk of dapsone hypersensitivity syndrome', *J Dermatol Sci,* 88(3), pp. 320-329.

Watkins, P. B., Lewis, J. H., Kaplowitz, N., Alpers, D. H., Blais, J. D., Smotzer, D. M., Krasa, H., Ouyang, J., Torres, V. E., Czerwiec, F. S. and Zimmer, C. A. (2015a) 'Clinical Pattern of Tolvaptan-Associated Liver Injury in Subjects with Autosomal Dominant Polycystic Kidney Disease: Analysis of Clinical Trials Database', *Drug Safety,* 38(11), pp. 1103-1113.

Watkins, P. B., Lewis, J. H., Kaplowitz, N., Alpers, D. H., Blais, J. D., Smotzer, D. M., Krasa, H., Ouyang, J., Torres, V. E., Czerwiec, F. S. and Zimmer, C. A. (2015b) 'Clinical Pattern of Tolvaptan-Associated Liver Injury in Subjects with Autosomal Dominant Polycystic Kidney Disease: Analysis of Clinical Trials Database', *Drug Saf,* 38(11), pp. 1103-13.

Wedemeyer, J., Tsai, M. and Galli, S. J. (2000) 'Roles of mast cells and basophils in innate and acquired immunity', *Curr Opin Immunol,* 12(6), pp. 624-31.

Wei, C. Y., Chung, W. H., Huang, H. W., Chen, Y. T. and Hung, S. I. (2012) 'Direct interaction between HLA-B and carbamazepine activates T cells in patients with Stevens-Johnson syndrome', *J Allergy Clin Immunol,* 129(6), pp. 1562-9.e5.

Werner, J. P. and Stephen, W. (2014) 'Interaction of Small Molecules with Specific Immune Receptors: The p-i Concept and its Consequences', *Current Immunology Reviews,* 10(1), pp. 7-18.

Whitaker, P., Meng, X., Lavergne, S. N., El-Ghaiesh, S., Monshi, M., Earnshaw, C., Peckham, D., Gooi, J., Conway, S., Pirmohamed, M., Jenkins, R. E., Naisbitt, D. J. and Park, B. K. (2011) 'Mass spectrometric characterization of circulating and functional antigens derived from piperacillin in patients with cystic fibrosis', *J Immunol,* 187(1), pp. 200-11.

WHO (2017) 'Hepatitis C', (Global hepatitis report, 2017).

Wieczorek, M., Abualrous, E. T., Sticht, J., Álvaro-Benito, M., Stolzenberg, S., Noé, F. and Freund, C. (2017) 'Major Histocompatibility Complex (MHC) Class I and MHC Class II Proteins: Conformational Plasticity in Antigen Presentation', *Frontiers in Immunology,* 8, pp. 292.

Williams, D. P., Kitteringham, N. R., Naisbitt, D. J., Pirmohamed, M., Smith, D. A. and Park, B. K. (2002) 'Are chemically reactive metabolites responsible for adverse reactions to drugs?', *Curr Drug Metab,* 3(4), pp. 351-66.

Willinger, T., Freeman, T., Hasegawa, H., McMichael, A. J. and Callan, M. F. (2005) 'Molecular signatures distinguish human central memory from effector memory CD8 T cell subsets', *J Immunol,* 175(9), pp. 5895-903.

Wilson, M. S. and Wynn, T. A. (2009) 'Pulmonary fibrosis: pathogenesis, etiology and regulation', *Mucosal immunology,* 2(2), pp. 103-121.

World Health Organization, W. (1966) ' International Drug Monitoring: The Role of the Hospital.', *Technical Report Series* No. 425.

Wu, C., Bell, C. M. and Wodchis, W. P. (2012) 'Incidence and Economic Burden of Adverse Drug Reactions among Elderly Patients in Ontario Emergency Departments', *Drug Safety,* 35(9), pp. 769-781.

Wu, X., Tian, J. and Wang, S. (2018) 'Insight Into Non-Pathogenic Th17 Cells in Autoimmune Diseases', *Frontiers in immunology,* 9, pp. 1112-1112.

Wu, Y., Farrell, J., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2007) 'Generation and characterization of antigen-specific CD4+, CD8+, and CD4+CD8+ T-cell clones from patients with carbamazepine hypersensitivity', *J Allergy Clin Immunol,* 119(4), pp. 973-81.

Wu, Y., Sanderson, J. P., Farrell, J., Drummond, N. S., Hanson, A., Bowkett, E., Berry, N., Stachulski, A. V., Clarke, S. E., Pichler, W. J., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2006) 'Activation of T cells by carbamazepine and carbamazepine metabolites', *J Allergy Clin Immunol,* 118(1), pp. 233-41.

Xia, M., Hu, S., Fu, Y., Jin, W., Yi, Q., Matsui, Y., Yang, J., McDowell, M. A., Sarkar, S., Kalia, V. and Xiong, N. (2014) 'CCR10 regulates balanced maintenance and function of resident regulatory and effector T cells to promote immune homeostasis in the skin', *The Journal Of Allergy And Clinical Immunology,* 134(3), pp. 634-644.e10.

Yadav, M., Louvet, C., Davini, D., Gardner, J. M., Martinez-Llordella, M., Bailey-Bucktrout, S., Anthony, B. A., Sverdrup, F. M., Head, R., Kuster, D. J., Ruminski, P., Weiss, D., Von Schack, D. and Bluestone, J. A. (2012) 'Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo', *The Journal of Experimental Medicine*.

Yamada, I., Suzuki, F., Kamiya, N., Aoki, K., Sakurai, Y., Kano, M., Matsui, H. and Kumada, H. (2012) 'Safety, pharmacokinetics and resistant variants of telaprevir alone for 12 weeks in hepatitis C virus genotype 1b infection', *Journal Of Viral Hepatitis,* 19(2), pp. e112-e119.

Yamashita, Y. i., Imai, K., Mima, K., Nakagawa, S., Hashimoto, D., Chikamoto, A. and Baba, H. (2017) 'Idiosyncratic drug-induced liver injury: A short review', *Hepatology Communications,* 1(6), pp. 494-500.

Yang, J., Zhang, L., Yu, C., Yang, X. F. and Wang, H. (2014) 'Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases', *Biomark Res,* 2, pp. 1.

Yaseen, F. S., Saide, K., Kim, S. H., Monshi, M., Tailor, A., Wood, S., Meng, X., Jenkins, R., Faulkner, L., Daly, A. K., Pirmohamed, M., Park, B. K. and Naisbitt, D. J. (2015) 'Promiscuous T-cell responses to drugs and drug-haptens', *J Allergy Clin Immunol*.

Yen, H. R., Harris, T. J., Wada, S., Grosso, J. F., Getnet, D., Goldberg, M. V., Liang, K. L., Bruno, T. C., Pyle, K. J., Chan, S. L., Anders, R. A., Trimble, C. L., Adler, A. J., Lin, T. Y., Pardoll, D. M., Huang, C. T. and Drake, C. G. (2009) 'Tc17 CD8 T cells: functional plasticity and subset diversity', *J Immunol,* 183(11), pp. 7161-8.

Yokoyama, W. M., Kim, S. and French, A. R. (2004) 'The dynamic life of natural killer cells', *Annu Rev Immunol,* 22, pp. 405-29.

Yoon, E., Babar, A., Choudhary, M., Kutner, M. and Pyrsopoulos, N. (2016) 'Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update', *Journal of clinical and translational hepatology,* 4(2), pp. 131-142.

You, Q., Cheng, L. and Ju, C. (2010) 'Generation of T cell responses targeting the reactive metabolite of halothane in mice', *Toxicology Letters,* 194(3), pp. 79-85.

Yun, J., Cai, F., Lee, F. J. and Pichler, W. J. (2016) 'T-cell-mediated drug hypersensitivity: immune mechanisms and their clinical relevance', *Asia Pacific Allergy,* 6(2), pp. 77-89.

Yun, J., Marcaida, M. J., Eriksson, K. K., Jamin, H., Fontana, S., Pichler, W. J. and Yerly, D. (2014) 'Oxypurinol directly and immediately activates the drug-specific T cells via the preferential use of HLA-B*58:01', *J Immunol,* 192(7), pp. 2984-93.

Zaid, A., Hor, J. L., Christo, S. N., Groom, J. R., Heath, W. R., Mackay, L. K. and Mueller, S. N. (2017) 'Chemokine Receptor-Dependent Control of Skin Tissue-Resident Memory T Cell Formation', *Journal Of Immunology (Baltimore, Md.: 1950),* 199(7), pp. 2451-2459.

Zanni, M. P., von Greyerz, S., Schnyder, B., Brander, K. A., Frutig, K., Hari, Y., Valitutti, S. and Pichler, W. J. (1998a) 'HLA-restricted, processing- and metabolism-independent pathway of drug recognition by human alpha beta T lymphocytes', *J Clin Invest,* 102(8), pp. 1591-8.

Zanni, M. P., von Greyerz, S., Schnyder, B., Wendland, T. and Pichler, W. J. (1998b) 'Allele-unrestricted presentation of lidocaine by HLA-DR molecules to specific alphabeta+ T cell clones', *Int Immunol,* 10(4), pp. 507-15.

Zarling, A. L., Luckey, C. J., Marto, J. A., White, F. M., Brame, C. J., Evans, A. M., Lehner, P. J., Cresswell, P., Shabanowitz, J., Hunt, D. F. and Engelhard, V. H. (2003) 'Tapasin is a facilitator, not an editor, of class I MHC peptide binding', *J Immunol,* 171(10), pp. 5287-95.

Zhang, B., Li, Q., Shi, C. and Zhang, X. (2018) 'Drug-Induced Pseudoallergy: A Review of the Causes and Mechanisms', *Pharmacology,* 101(1-2), pp. 104-110.

Zhang, F. R., Liu, H., Irwanto, A., Fu, X. A., Li, Y., Yu, G. Q., Yu, Y. X., Chen, M. F., Low, H. Q., Li, J. H., Bao, F. F., Foo, J. N., Bei, J. X., Jia, X. M., Liu, J., Liany, H., Wang, N., Niu, G. Y., Wang, Z. Z., Shi, B. Q., Tian, H. Q., Liu, H. X., Ma, S. S., Zhou, Y., You, J. B., Yang, Q., Wang, C., Chu, T. S., Liu, D. C., Yu, X. L., Sun, Y. H., Ning, Y., Wei, Z. H., Chen, S. L., Chen, X. C., Zhang, Z. X., Liu, Y. X., Pulit, S. L., Wu, W. B., Zheng, Z. Y., Yang, R. D., Long, H., Liu, Z. S., Wang, J. Q., Li, M., Zhang, L. H., Wang, H., Wang, L. M., Xiao, P., Li, J. L., Huang, Z. M., Huang, J. X., Li, Z., Liu, J., Xiong, L., Yang, J., Wang, X. D., Yu, D. B., Lu, X. M., Zhou, G. Z., Yan, L. B., Shen, J. P., Zhang, G. C., Zeng, Y. X., de Bakker, P. I., Chen, S. M. and Liu, J. J. (2013) 'HLA-B*13:01 and the dapsone hypersensitivity syndrome', *N Engl J Med,* 369(17), pp. 1620-8.

Zhang, J.-M. and An, J. (2007) 'Cytokines, Inflammation and Pain', *International anesthesiology clinics,* 45(2), pp. 27-37.

Zhang, Q. and Vignali, Dario A. A. (2016) 'Co-stimulatory and Co-inhibitory Pathways in Autoimmunity', *Immunity,* 44(5), pp. 1034-1051.

Zhao, Y., Niu, C. and Cui, J. (2018) 'Gamma-delta (γδ) T cells: friend or foe in cancer development?', *Journal of Translational Medicine,* 16(1), pp. 3.

Zhou, L., Ivanov, II, Spolski, R., Min, R., Shenderov, K., Egawa, T., Levy, D. E., Leonard, W. J. and Littman, D. R. (2007) 'IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways', *Nat Immunol,* 8(9), pp. 967-74.

Zou, Y.-R., Grimaldi, C. and Diamond, B. (2017) 'Chapter 13 - B Cells A2 - Firestein, Gary S', in Budd, R.C., Gabriel, S.E., McInnes, I.B. & O'Dell, J.R. (eds.) *Kelley and Firestein's Textbook of Rheumatology (Tenth Edition)*: Elsevier, pp. 207-230.e3.

Zuniga, F. I., Loi, D., Ling, K. H. J. and Tang-Liu, D. D. S. (2012) 'Idiosyncratic reactions and metabolism of sulfur-containing drugs', *Expert Opinion on Drug Metabolism & Toxicology,* 8(4), pp. 467-485.